PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/29, 15/62, 15/70, 15/86, A61K 38/16

A1

(11) International Publication Number: WO 97/41233

(43) International Publication Date: 6 November 1997 (06.11.97)

(21) International Application Number: PCT/CA97/00288

(22) International Filing Date: 29 April 1997 (29.04.97)

(30) Priority Data:
60/016,509 30 April 1996 (30.04.96) US

(71) Applicant *(for all designated States except US)*: CANGENE CORPORATION [CA/CA]; 104 Chancellor Matheson Road, Winnipeg, Manitoba R3T 2N2 (CA).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: BORGFORD, Thor [CA/CA]; 443 Fader Street, New Westminster, British Columbia V3L 3T2 (CA).

(74) Agent: BERESKIN & PARR; 40th floor, 40 King Street West, Toronto, Ontario M5H 3Y2 (CA).

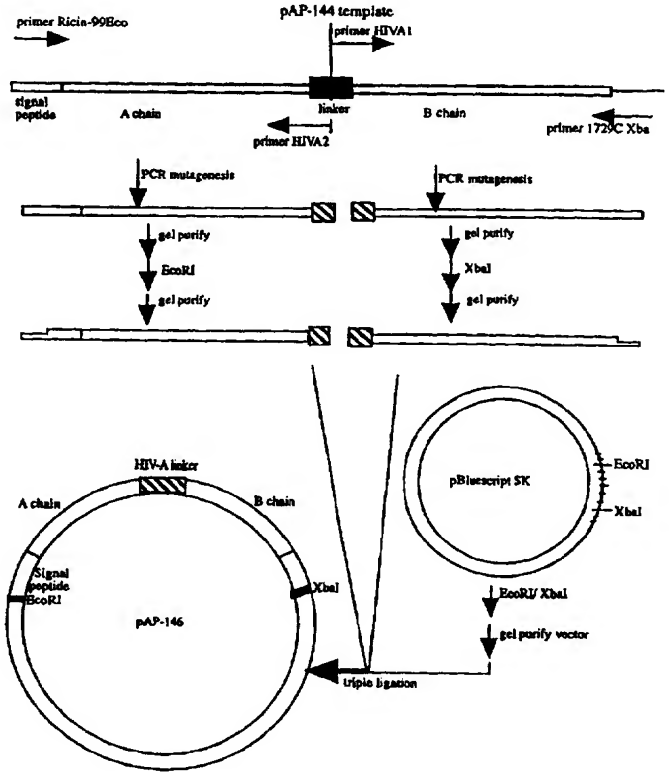
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ANTIVIRAL RICIN-LIKE PROTEINS

(57) Abstract

The present invention provides a protein having an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains. The linker sequence contains a cleavage recognition site for a retroviral protease such as HIV or an HTLV protease. The invention also relates to a nucleic acid molecule encoding the protein and to expression vectors incorporating the nucleic acid molecule. Also provided is a method of inhibiting or destroying mammalian cells infected with a retrovirus utilizing the proteins of the invention; and pharmaceutical compositions for treating HIV infections and human T-cell leukemias involving HTLV.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

Title: Antiviral Ricin-Like Proteins

FIELD OF THE INVENTION

The invention relates to proteins having A and B chains of a ricin-like toxin, linked by a linker sequence which is specifically cleavable by a retroviral protease to release the active A chain. The invention also relates to a nucleic acid molecule encoding the protein and to expression vectors incorporating the nucleic acid molecule. Also provided is a method of inhibiting or destroying mammalian cells infected with a retrovirus utilizing the proteins of the invention and pharmaceutical compositions for treating HIV infection.

BACKGROUND OF THE INVENTION

Bacteria and plants are known to produce cytotoxic proteins which may consist of one, two or several polypeptides or subunits. Those proteins having a single subunit may be loosely classified as Type I proteins. Many of the cytotoxins which have evolved two subunit structures are referred to as type II proteins(Saelinger, C.B. in *Trafficking of Bacterial Toxins* (eds. Saelinger, C.B.) 1-13 (CRC Press Inc., Boca Raton, Florida, 1990). One subunit, the A chain, possesses the toxic activity whereas the second subunit, the B chain, binds cell surfaces and mediates entry of the toxin into a target cell. A subset of these toxins kill target cells by inhibiting protein biosynthesis. For example, bacterial toxins such as diphtheria toxin or *Pseudomonas* exotoxin inhibit protein synthesis by inactivating elongation factor 2. Plant toxins such as ricin work by directly inactivating ribosomes [Olsnes, S. & Phil, A. in *Molecular action of toxins and viruses* (eds. Cohen, P. & vanHeyningen, S.); 51-105 (Elsevier Biomedical Press, Amsterdam, 1982].

Ricin, derived from the seeds of *Ricinus communis* (castor oil plant), is the most potent of the plant toxins. It is estimated that a single ricin A chain is able to inactivate ribosomes at a rate of 1500 ribosomes/minute. Consequently, a single molecule of ricin is enough to kill a cell (Olsnes, S. & Phil, A. in *Molecular action of toxins and viruses* (eds. Cohen, P. & vanHeyningen, S.) 51-105 (Elsevier Biomedical Press, Amsterdam, 1982). The ricin toxin is a glycosylated heterodimer with A and B chain molecular masses of 30,625 Da and 31,431 Da respectively. The A chain of ricin has an N-glycosidase activity and catalyzes the excision of a specific adenine residue from the 28S rRNA of eukaryotic ribosomes (Endo, Y; & Tsurugi, K. J. *Biol. Chem.* 262:8128 (1987)). The B chain of ricin, although not toxic in itself, promotes the toxicity of the A chain by binding to galactose residues on the surface of eukaryotic cells and stimulating receptor-mediated endocytosis of the toxin molecule (Simmons et al. *Biol. Chem.* 261:7912 (1986)).

Protein toxins are initially produced in an inactive, precursor form. Ricin is initially produced as a single polypeptide (preproricin) with a 35 amino acid N-terminal

presequence and 12 amino acid linker between the A and B chains. The pre-sequence is removed during translocation of the ricin precursor into the endoplasmic reticulum (Lord, J.M. *Eur. J. Biochem.* 146:403-409 (1985) and Lord, J.M. *Eur. J. Biochem.* 146:411-416 (1985)). The proricin is then translocated into specialized organelles called protein bodies where a plant protease cleaves the protein at a linker region between the A and B chains (Lord, J.M. et al., *FASAB Journal* 8:201-208 (1994)). The two chains, however, remain covalently attached by an interchain disulfide bond (cysteine 259 in the A chain to cysteine 4 in the B chain) and mature disulfide linked ricin is secreted from the plant cells. The A chain is inactive in the proricin (O'Hare, M., *et al. FEBS Lett.* 273:200-204 (1990)) and it is inactive in the disulfide-linked mature ricin(Richardson, P.T., *et al. FEBS Lett.* 255:15-20 (1989)). The ribosomes of the castor bean plant are themselves susceptible to inactivation by ricin A chain; however, as there is no cell surface galactose to permit B chain recognition the A chain cannot re-enter the cell. The exact mechanism of A chain release and activation in target cell cytoplasm is not known (Lord, J.M. et al., *FASAB Journal* 8:201-208 (1994)). However, it is known that for activation to take place the disulfide bond between the A and B chains must be reduced and, hence, the linkage between subunits broken.

The ricin gene has been cloned and sequenced, and the X-ray crystal structures of the A and B chains have been described (Rutenber, E., et al. Proteins 10:240-250 (1991); Weston et al., Mol. Bio. 244:410-422, 1994; Lamb and Lord *Eur. J. Biochem.* 14:265 (1985); Halling, K., *et al. Nucleic Acids Res.* 13:8019 (1985)). Due to its extreme toxicity there has been much interest in making ricin-based immunotoxins as therapeutic agents for destroying or inhibiting target cells or organisms (Vitetta et al., *Science* 238:1098-1104(1987)). An immunotoxin is a conjugate of a specific cell-binding component, such as a monoclonal antibody or growth factor and the toxin in which the two protein components are covalently linked. Generally, the components are chemically coupled. However, the linkage may also be a peptide or disulfide bond. The antibody directs the toxin to cell types presenting a specific antigen thereby providing a specificity of action not possible with the natural toxin. Immunotoxins have been made both with the entire ricin molecule (i.e. both chains) and with the ricin A chain alone (Spooner et al. *Mol. Immunol.* 31:117-125, (1994)).

Immunotoxins made with the ricin dimer (IT-Rs) are more potent toxins than those made with only the A chain (IT-As). The increased toxicity of IT-Rs is thought to be attributed to the dual role of the B chains in binding to the cell surface and in translocating the A chain to the cytosolic compartment of the target cell (Vitetta et al., *Science* 238:1098-1104(1987); Vitetta & Thorpe *Seminars in Cell Biology* 2:47-58 (1991)). However, the presence of the B chain in these conjugates also promotes the entry of the

immunotoxin into nontarget cells. Even small amounts of B chain may override the specificity of the cell-binding component as the B chain binds nonspecifically to N-glycosylated galactose, present on most cells. IT-As are more specific and safer to use than IT-Rs. However, in the absence of the B chain the A chain has greatly reduced toxicity.

A number of immunotoxins have been designed to recognize antigens on the surfaces of tumour cells. A major problem with the use of ITs is that often the target antigen is also found on non-tumour cells (Vitetta et al., *Immunology Today* 14:252-259 (1993)). Also, due to the reduced potency of IT-As as compared to ITRs, large doses of IT-As must be administered to patients. The large doses frequently cause immune responses and production of neutralizing antibodies in patients (Vitetta et al., *Science* 238:1098-1104(1987)). IT-As and IT-Rs both suffer from reduced toxicity as the A chain is not released from the conjugate into the target cell cytoplasm.

The insertion of intramolecular cleavage sites between the cytotoxic and cell-binding components of a toxin can mimic the way that the natural toxin is activated. European patent application no. 466,222 describes the use of maize-derived pro-proteins which can be converted into active form by cleavage with extracellular blood enzymes such as factor Xa, thrombin or collagenase. Westby et al. (Bioconjugate Chem., 3:375-381, 1992) documented fusion proteins which have a specific cell binding component and proricin with a protease sensitive cleavage site specific for factor Xa within the linker sequence. O'Hare et al. (FEBS Lett. 273:200-204, 1990) also describe a recombinant fusion protein of RTA and staphylococcal protein A joined by a trypsin-sensitive cleavage site. In view of the prevalence of the extracellular proteases utilized in these approaches, such artificial activation of the toxin precursor or immunotoxin do not confer a mechanism for intracellular toxin activation, and the problems of target specificity and adverse immunological reactions to the cell-binding component of the immunotoxin remain.

In view of the extreme toxicity of proteins such as ricin, the lack of specificity of the immunotoxins may severely limit their usefulness as therapeutics for the treatment of cancer and infectious diseases. The preparation of a suitable specific cell binding component may be problematic. For example, antigens specific for the target cell may not be available and many potential target cells and infective organisms can alter their antigenic make up rapidly to avoid immune recognition.

The potential of bacterial and plant toxins for inhibiting mammalian retroviruses, particularly AIDS, has been investigated. Bacterial toxins such as *Pseudomonas* exotoxin-A and subunit A of diphtheria toxin; dual chain ribosomal inhibitory plant toxins, such as ricin and single chain ribosomal inhibitory proteins such as trichosanthin and poke weed antiviral protein have been used for the elimination of HIV infected cells (Olson et al. 1991, AIDS Res. and Human Retroviruses 7:1025-1030). The high toxicity of

these toxins for mammalian cells, combined with a lack of specificity of action poses a major problem to the development of pharmaceuticals incorporating the toxins, such as immunotoxins.

Immunotoxins are designed such that their specificity of action is determined solely by the antibody component; antigen presenting cells are preferentially destroyed by the drug (Pastan et al., *Annals New York Academy of Sciences* 758:345-353 (1995)). The toxin protein of immunotoxin conjugates does not give the therapeutic any additional specificity of action; it will bring about the destruction of any cell it is delivered to.

SUMMARY OF THE INVENTION

The present inventors have prepared novel recombinant toxic proteins which are specifically toxic to cells infected with retroviruses and which do not depend for their specificity of action on a specific cell-binding component. The recombinant proteins of the invention have an A chain of a ricin-like toxin linked to a B chain by a linker sequence, which may be specifically cleaved by a retroviral protease within infected cells to activate the toxic A chain.

In one aspect, the present invention provides a purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterlogous linker amino acid sequence, linking the A and B chains. The linker sequence is not a linker sequence of a ricin-like toxin, but rather the heterologous linker sequence contains a cleavage recognition site for a retroviral protease. The A and or the B chain may be those of ricin.

In an embodiment, the cleavage recognition site is the cleavage recognition site for an HIV protease. In a particular embodiment, the linker amino acid sequence comprises VSQNYPIVQNFN; SKARVLAEAMSN; or SIRKILFLDGIN. In further particular embodiments, the nucleic acid has the nucleotide sequence shown in Figure 8, Figure 9 or Figure 10.

In another embodiment, the cleavage recognition site is the cleavage recognition site for a human T-cell leukemia virus protease. In a particular embodiment, the linker amino acid sequence comprises SAPQVLPVMHPN or SKTKVLVVQPKN cleaved by a human T-cell leukemia virus-I (HTLV-I) protease; or, SKTKVLVVQPRN or STTQCFPILHPN cleaved by a human T-cell leukemia virus-II (HTLV-II) protease.

The present invention further provides a plasmid incorporating the nucleic acid of the invention. In an embodiment, the plasmid has the restriction map as shown in Figure 1A, 2A, 3A, 16A, 17A, 18A, or 19A.

In another embodiment, the present invention provides a baculovirus transfer vector incorporating the nucleic acid of the invention. In particular embodiments, the invention provides a baculovirus transfer vector having the restriction map as shown in

Figures 5, 6, 7, 16C, 17C, 18C, or 19C or having the DNA sequence as shown in Figure 11.

In a further aspect, the present invention provides a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin- like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for a retroviral protease. The A and or the B chain may be those of ricin.

In another aspect, the invention provides a method of inhibiting or destroying mammalian cells infected with a retrovirus having a protease, comprising the steps of preparing a recombinant protein of the invention having a heterologous linker sequence which contains a cleavage recognition site for the retrovirus protease and introducing the recombinant protein into the cells. In an embodiment, the retrovirus is HIV.

The present invention also relates to a method of treating a mammal infected with HIV by administering the recombinant proteins of the invention to the mammal.

Also provided is a process for preparing a pharmaceutical for treating a mammal infected with a retrovirus having a protease comprising the steps of preparing a purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for the protease; introducing the nucleic acid into a host cell; expressing the nucleic acid in the host cell to obtain a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterlogous linker amino acid sequence, linking the A and B chains wherein the linker sequence contains the cleavage recognition site for the protease; and suspending the protein in a pharmaceutically acceptable carrier, diluent or excipient.

In an embodiment, a process is provided for preparing a pharmaceutical for treating a mammal infected with a retrovirus having a protease comprising the steps of identifying a cleavage recognition site for the protease; preparing a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterlogous linker amino acid sequence, linking the A and B chains wherein the linker sequence contains the cleavage recognition site for the protease and suspending the protein in a pharmaceutically acceptable carrier, diluent or excipient.

In a further aspect, the invention provides a pharmaceutical composition for treating a retroviral infection, such as HIV, in a mammal comprising the recombinant protein of the invention and a pharmaceutically acceptable carrier, diluent or excipient.

The invention also contemplates a method for treating cancer cells containing an HTLV protease comprising (a) preparing a recombinant protein of the invention having a heterologous linker sequence which contains a cleavage recognition site for an HTLV

protease; an (b) introducing the recombinant protein into the cells. The method can be used to treat a mammal with human T-cell leukemias involving HTLV. Compositions for treating human T-cell leukemias involving HTLV comprising the recombinant protein of the invention having a heterologous linker sequence which contains a cleavage recognition site for an HTLV protease, and a pharmaceutically acceptable carrier , diluent, or excipient are also provided.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

DESCRIPTION OF THE DRAWINGS

The invention will be better understood with reference to the drawings in which:

Figure 1A summarizes the cloning strategy used to generate the pAP-146 construct;

Figure 1B shows the nucleotide sequence of the HIV-A linker region of pAP-146;

Figure 2A summarizes the cloning strategy used to generate the pAP-147 construct;

Figure 2B shows the nucleotide sequence of the HIV-B linker region of pAP-147;

Figure 3A summarizes the cloning strategy used to generate the pAP-148 construct;

Figure 3B shows the nucleotide sequence of the HIV-H linker region of pAP-148;

Figure 4 shows the amino acid sequences of the wild type ricin linker, the pAP-146 linker, the pAP-147 linker and the pAP-148 linker;

Figure 5 shows the subcloning of the HIV-A linker variant into a baculovirus transfer vector;

Figure 6 shows the subcloning of the HIV-B linker variant into a baculovirus transfer vector;

Figure 7 shows the subcloning of the HIV-H linker variant into a baculovirus transfer vector;

Figure 8 shows the DNA sequence of the pAP-190 insert;

Figure 9 shows the DNA sequence of the pAP-196 insert;

Figure 10 shows the DNA sequence of the pAP-197 insert;

Figure 11 shows the DNA sequence of the baculovirus transfer vector pVL1393;

Figure 12 is a diagram of the vector pSB2;

Figure 13 shows a Western Blot of a pAP-190 proricin variant;

Figure 14 is a blot showing cleavage of a pAp 190 proricin variant by HIV protease;

Figure 15 is a blot showing activation of pAP-190 proricin variant by HIV

protease;

Figure 16A is a diagram summarizing the cloning strategy used to generate the pAP-205 construct;

Figure 16B shows the nucleotide sequence of the HTLV-I-A linker regions of pAP-205;

Figure 16C is a diagram showing the subcloning of the HTLV-I-A linker variant into a baculovirus transfer vector;

Figure 16D shows the DNA sequence of the pAP-206 insert containing ricin and the HTLV-I-A linker;

Figure 17A is a diagram summarizing the cloning strategy used to generate the pAP-207 construct;

Figure 17B shows the nucleotide sequence of the HTLV-I-B linker regions of pAP-207;

Figure 17C is a diagram summarizing the subcloning of the HTLV-I-B linker variant into a baculovirus transfer vector;

Figure 17D shows the DNA sequence of the pAP-208 insert containing ricin and the HTLV-I-B linker;

Figure 18A is a diagram summarizing the cloning strategy used to generate the pAP-209 construct;

Figure 18B shows the nucleotide sequence of the HTLV-II-A linker regions of pAP-209;

Figure 18C is a diagram summarizing the subcloning of the HTLV-II-A linker variant into a baculovirus transfer vector;

Figure 18D shows the DNA sequence of the pAP-210 insert containing ricin and the HTLV-II-A linker;

Figure 19A is a diagram summarizing the cloning strategy used to generate the pAP-211 construct;

Figure 19B shows the nucleotide sequence of the HTLV-II-B linker regions of pAP-211;

Figure 19C is a diagram summarizing the subcloning of the HTLV-II-B linker variant into a baculovirus transfer vector;

Figure 19D shows the DNA sequence of the pAP-212 insert containing ricin and the HTLV-II-B linker; and

Figure 20 shows the amino acid sequences of the wild type ricin linker and HTLV protease-sensitive amino acid linkers contained in linkers pAP-205 to pAP-212.

DETAILED DESCRIPTION OF THE INVENTION

Nucleic Acid Molecules of the Invention

The present inventors have cloned and expressed novel nucleic acid molecules having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains. The heterologous linker sequence contains a cleavage recognition site for a retroviral protease such as a cleavage recognition site for HIV or a human T-cell leukemia virus protease.

The term "isolated and purified" as used herein refers to a nucleic acid substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical precursors, or other chemicals when chemically synthesized. An "isolated and purified" nucleic acid is also substantially free of sequences which naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) from which the nucleic acid is derived. The term "nucleic acid" is intended to include DNA and RNA and can be either double stranded or single stranded.

The term "linker sequence" as used herein refers to an internal amino acid sequence within the protein encoded by the nucleic acid molecule of the invention which contains residues linking the A and B chain so as to render the A chain incapable of exerting its toxic effect, for example catalytically inhibiting translation of a eukaryotic ribosome. By heterologous is meant that the linker sequence is not a sequence native to the A or B chain of a ricin-like toxin or precursor thereof. However, preferably, the linker sequence may be of a similar length to the linker sequence of a ricin-like toxin and should not interfere with the role of the B chain in cell binding and transport into the cytoplasm. When the linker sequence is cleaved the A chain becomes active or toxic.

The nucleic acid molecule of the invention was cloned by subjecting a preproricin cDNA clone (pAP-144) to site-directed mutagenesis in order to generate a series of variants differing only in the sequence between the A and B chains (linker region). Oligonucleotides, corresponding to the extreme 5' and 3' ends of the preproricin gene were synthesized and used to PCR amplify the gene. Using the cDNA sequence for preproricin (Lamb et al., Eur. J. Biochem., 145:266-270, 1985), several oligonucleotide primers were designed to flank the start and stop codons of the preproricin open reading frame.

The preproricin cDNA was amplified using the upstream primer Ricin-99 (or Ricin-109 may be used) and the downstream primer Ricin1729C with Vent DNA polymerase (New England Biolabs) using standard procedures (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)). The use of the upstream primer Ricin-109 circumvents the subcloning step into vector pSB2. The purified PCR fragment encoding the preproricin cDNA was then ligated into an Eco RV-digested pBluescript II SK plasmid (Stratagene), and used to transform competent XL1-Blue cells (Stratagene).

The cloned PCR product containing the putative preproricin gene was confirmed by

DNA sequencing of the entire cDNA clone (pAP-144). The sequences and location of oligonucleotide primers used for sequencing are shown in Table 1.

The preproricin cDNA clone (pAP-144) was subjected to site-directed mutagenesis in order to generate a series of variants differing only in the sequence between the A and B chains (linker region). The wild-type preproricin linker region was replaced with the three linker sequences, pAP-146, pAP-147 and pAP-148 shown in Figure 4. The linker regions of the variants encode an HIV protease cleavage recognition sequence (Slalka et al., Cell, 56:911-913, 1989). The mutagenesis and cloning strategy used to generate the linker variants pAP-146, pAP-147 and pAP-148 are summarized in Figures 1A and 1B, 2A and 2B and 3A and 3B respectively. The first step involved a DNA amplification using a set of mutagenic primers (HIVA 1, 2; HIVB 1, 2; HIVH 1, 2) in combination with the two flanking primers Ricin-99Eco and Ricin1729Xba. Restriction digested PCR fragments were gel purified and then ligated with PBluescript SK which had been digested with Eco RI and Xba I. Ligation reactions were used to transform competent XL1-Blue cells (Stratagene). Recombinant clones were identified by restriction digests of plasmid miniprep DNA and the mutant linker sequences were confirmed by DNA sequencing.

Recombinant clones were subcloned into vector pSB2. The three constructs obtained were pAP-151, pAP-159, and pAP-163, with each having the mutant linker found in pAP-146, pAP-147, and pAP-148 respectively.

The cloning strategy described above may also be applied to the preparation of recombinant clones containing a cleavage recognition site for a human T-cell leukemia virus protease. For example, recombinant clones pAP-205, pAP-207, pAP-209, and pAP-211 were prepared using a method similar to the one described above.

The nucleic acid molecule of the invention has sequences encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker sequence containing a cleavage recognition site for a retroviral protease, such as an HIV protease or an HTLV protease. The nucleic acid may be expressed to provide a recombinant protein having an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker sequence containing a cleavage recognition site for a retroviral protease, such as an HIV protease or an HTLV protease.

The nucleic acid molecule may comprise the A and/or B chain of ricin. The ricin gene has been cloned and sequenced, and the X-ray crystal structures of the A and B chains are published (Rutenber, E., et al. Proteins 10:240-250 (1991); Weston et al., Mol. Bio. 244:410-422, 1994; Lamb and Lord *Eur. J. Biochem.* 14:265 (1985); Halling, K., *et al. Nucleic Acids Res.* 13:8019 (1985)). It will be appreciated that the invention includes nucleic acid molecules encoding truncations of A and B chains of ricin-like proteins and analogs and homologs of A and B chains of ricin-like proteins and truncations thereof (i.e.,ricin-like

proteins), as described herein. It will further be appreciated that variant forms of the nucleic acid molecules of the invention which arise by alternative splicing of an mRNA corresponding to a cDNA of the invention are encompassed by the invention.

Another aspect of the invention provides a nucleotide sequence which hybridizes under high stringency conditions to a nucleotide sequence encoding the A and/or B chains of a ricin-like protein. Appropriate stringency conditions which promote DNA hybridization are known to those skilled in the art, or can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C may be employed. The stringency may be selected based on the conditions used in the wash step. By way of example, the salt concentration in the wash step can be selected from a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be at high stringency conditions, at about 65°C.

The nucleic acid molecule may comprise the A and/or B chain of a ricin-like toxin. Methods for cloning ricin-like toxins are known in the art and are described, for example, in E.P. 466,222. Sequences encoding ricin or ricin-like A and B chains may be obtained by selective amplification of a coding region, using sets of degenerative primers or probes for selectively amplifying the coding region in a genomic or cDNA library. Appropriate primers may be selected from the nucleic acid sequence of A and B chains of ricin or ricin-like toxins. It is also possible to design synthetic oligonucleotide primers from the nucleotide sequences for use in PCR. Suitable primers may be selected from the sequences encoding regions of ricin-like proteins which are highly conserved, as described for example in U.S. Patent No 5,101,025 and E.P. 466,222.

A nucleic acid can be amplified from cDNA or genomic DNA using these oligonucleotide primers and standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. It will be appreciated that cDNA may be prepared from mRNA, by isolating total cellular mRNA by a variety of techniques, for example, by using the guanidinium-thiocyanate extraction procedure of Chirgwin et al., Biochemistry, 18, 5294-5299 (1979). cDNA is then synthesized from the mRNA using reverse transcriptase (for example, Moloney MLV reverse transcriptase available from Gibco/BRL, Bethesda, MD, or AMV reverse transcriptase available from Seikagaku America, Inc., St. Petersburg, FL).

It will be appreciated that the methods described above may be used to obtain the coding sequence from plants, bacteria or fungi, preferably plants, which produce known ricin-like proteins and also to screen for the presence of genes encoding as yet unknown ricin-like proteins.

A sequence containing a cleavage recognition site for a retroviral protease may be

selected based on the retrovirus which is to be targeted by the recombinant protein. The cleavage recognition site may be selected from sequences known to encode a cleavage recognition site for the retrovirus protease. Sequences encoding cleavage recognition sites may be identified by testing the expression product of the sequence for susceptibility to cleavage by a retroviral protease. An assay to identify peptides having cleavage recognition sites for HIV protease is described in PCT/US88/01849. The HIV protease encoded by the p17 gene of HIV and has the highly conserved Asp-Thr-Gly sequence characteristic of the active site of cellular aspartyl proteases. The HIV protease may be prepared by methods known in the art and used to test suspected cleavage recognition sites. For example, a polypeptide containing the suspected cleavage recognition site may be incubated with the protease and the amount of cleavage product determined (DiIannit, 1990, J. Biol. Chem. 285: 17345-17354). Substrates for HIV proteases are described in U.S. Patent No. 5,235,039. The invention is not restricted to proteins including the cleavage recognition site for HIV proteases, but includes recognition sites of other retroviral proteases, including proteases of members of the subfamilies oncovirinae, lentivirinae and spumavirinae for example from HTLV, AMV, RSV, BLV, FeLV and MMTV. Examples of retroviral proteases and conserved sequences thereof are provided, for example, in Katoh et al., (Nature 329:654-656).

A sequence containing a cleavage recognition site for an HTLV protease may be selected using the conventional methods described herein. The preparation of human T-cell leukemia virus proteases, their substrates and enzymatic activity assay methodology have been described by Petit, S.C. et al. (J. Biol. Chem. 266:14539-14547 (1991)) and Blaha, I. et al. (FEBS Lett. 309:389-393 (1992)).

In an embodiment, the cleavage recognition site is the cleavage recognition site for an HIV protease. In a particular embodiment, the linker amino acid sequence comprises VSQNYPIVQNFN; SKARVLAEAMSN; or SIRKILFLDGIN. In further particular embodiments, the nucleic acid has the nucleotide sequence shown in Figure 8, Figure 9 or Figure 10.

In another embodiment, the cleavage recognition site is the cleavage recognition site for a human T-cell leukemia virus protease. In a particular embodiment, the linker amino acid sequence comprises SAPQVLPVMHPN or SKTKVLVVQPKN cleaved by a human T-cell leukemia virus-I (HTLV-I) protease; or, SKTKVLVVQPRN or STTQCFPILHPN cleaved by a human T-cell leukemia virus-II (HTLV-II) protease.

The nucleic acid molecule of the invention may be prepared by site directed mutagenesis. For example, the cleavage site of a retroviral protease may be prepared by site directed mutagenesis of the homologous linker sequence of a proricin-like toxin. Procedures for cloning proricin-like genes, encoding a linker sequence are described in EP

466,222. Site directed mutagenesis may be accomplished by DNA amplification of mutagenic primers in combination with flanking primers. Suitable procedures using the mutagenic primers HIVA1, HIVB1 and HIVH1 are shown in Figures 1A to 3B, and Figures 16A, 16B, 17A, 17B, 18A, 19A and 19B.

The nucleic acid molecule of the invention may also encode a fusion protein. A sequence encoding a heterologous linker sequence containing a cleavage recognition site for a retroviral protease may be cloned from a cDNA or genomic library or chemically synthesized based on the known sequence of such cleavage sites. The heterologous linker sequence may then be fused in frame with the sequences encoding the A and B chains of the ricin-like toxin for expression as a fusion protein. It will be appreciated that a nucleic acid molecule encoding a fusion protein may contain a sequence encoding an A chain and a B chain from the same ricin-like toxin or the encoded A and B chains may be from different toxins. For example, the A chain may be derived from ricin and the B chain may be derived from abrin. A protein may also be prepared by chemical conjugation of the A and B chains and linker sequence using conventional coupling agents for covalent attachment.

An isolated and purified nucleic acid molecule of the invention which is RNA can be isolated by cloning a cDNA encoding an A and B chain and a linker into an appropriate vector which allows for transcription of the cDNA to produce an RNA molecule which encodes a protein of the invention. For example, a cDNA can be cloned downstream of a bacteriophage promoter, (e.g. a T7 promoter) in a vector, cDNA can be transcribed in vitro with T7 polymerase, and the resultant RNA can be isolated by standard techniques.

Recombinant Protein of the Invention

As previously mentioned, the invention provides novel recombinant proteins which incorporate the A and B chains of a ricin-like toxin linked by a heterologous linker sequence containing a cleavage recognition site for a retroviral protease, such as an HIV protease or an HTLV protease. It is an advantage of the recombinant proteins of the invention that they are non-toxic until the A chain is liberated from the B chain by specific cleavage of the linker by the retroviral protease, such as an HIV protease or an HTLV protease. Thus the protein may be used to specifically target cells infected with the retrovirus in the absence of additional specific cell-binding components to target infected cells. It is a further advantage that the retroviral protease cleaves the heterologous linker intracellularly thereby releasing the toxic A chain directly into the cytoplasm of the infected cell. As a result, infected cells are specifically targeted and non-infected cells are not directly exposed to the activated free A chain.

Ricin is a plant derived ribosome inhibiting protein which blocks protein synthesis in eukaryotic cells. Ricin may be derived from the seeds of *Ricinus communis*

(castor oil plant).The ricin toxin is a glycosylated heterodimer with A and B chain molecular masses of 30,625 Da and 31,431 Da respectively. The A chain of ricin has an N-glycosidase activity and catalyzes the excision of a specific adenine residue from the 28S rRNA of eukaryotic ribosomes (Endo, Y; & Tsurugi, K. J. *Biol. Chem.* 262:8128 (1987)). The B chain of ricin, although not toxic in itself, promotes the toxicity of the A chain by binding to galactose residues on the surface of eukaryotic cells and stimulating receptor-mediated endocytosis of the toxin molecule (Simmons et al. *Biol. Chem.* 261:7912 (1986)).

Protein toxins are initially produced in an inactive, precursor form. Ricin is initially produced as a single polypeptide (preproricin) with a 35 amino acid N-terminal presequence and 12 amino acid linker between the A and B chains. The pre-sequence is removed during translocation of the ricin precursor into the endoplasmic reticulum (Lord, J.M. *Eur. J. Biochem.* 146:403-409 (1985) and Lord, J.M. *Eur. J. Biochem.* 146:411-416 (1985)). The proricin is then translocated into specialized organelles called protein bodies where a plant protease cleaves the protein at a linker region between the A and B chains (Lord, J.M. et al., *FASAB Journal* 8:201-208 (1994)). The two chains, however, remain covalently attached by an interchain disulfide bond (cysteine 259 in the A chain to cysteine 4 in the B chain) and mature disulfide linked ricin is secreted from the plant cells. The A chain is inactive in the proricin (O'Hare, M., *et al. FEBS Lett.* 273:200-204 (1990)) and it is inactive in the disulfide-linked mature ricin(Richardson, P.T., *et al. FEBS Lett.* 255:15-20 (1989)). The ribosomes of the castor bean plant are themselves susceptible to inactivation by ricin A chain; however, as there is no cell surface galactose to permit B chain recognition the A chain cannot re-enter the cell.

Ricin-like proteins include bacterial, fungal and plant toxins which have A and B chains and inactivate ribosomes and inhibit protein synthesis. The A chain is an active polypeptide subunit which is responsible for the pharmacologic effect of the toxin. In most cases the active component of the A chain is an enzyme. The B chain is responsible for binding the toxin to the cell surface and is thought to facilitate entry of the A chain into the cell cytoplasm. The A and B chains in the mature toxins are linked by disulfide bonds. The toxins most similar in structure to ricin are plant toxins which have one A chain and one B chain. Examples of such toxins include abrin which may be isolated from the seeds of *Abrus precatorius*, ricin which may be isolated from the seeds of castor beans *Ricinus communis*, and modeccin.

Ricin-like bacterial proteins include diphtheria toxin, which is produced by *Corynebacterium diphtheriae*, *Pseudomonas* enterotoxin A and cholera toxin. It will be appreciated that the term ricin-like toxins is also intended to include the A chain of those toxins which have only an A chain. The recombinant proteins of the invention could include the A chain of these toxins conjugated to, or expressed as, a recombinant protein

with the B chain of another toxin. Examples of plant toxins having only an A chain include trichosanthin, MMC and pokeweed antiviral proteins, dianthin 30, dianthin 32, crotin II, curcin II and wheat germ inhibitor. Examples of fungal toxins having only an A chain include alpha-sarcin, restrictocin, mitogillin, enomycin, phenomycin. Examples of bacterial toxins having only an A chain include cytotoxin from *Shigella dysenteriae* and related Shiga-like toxins. Recombinant trichosanthin and the coding sequence thereof is disclosed in U.S. Patents Nos. 5,101,025 and 5,128,460.

In addition to the entire B or A chains of a ricin-like toxin, it will be appreciated that the recombinant protein of the invention may contain only that portion of the A chain which is necessary for exerting its cytotoxic effect. For example, the first 30 amino acids of the ricin A chain may be removed resulting in a truncated A chain which retains toxic activity. The truncated ricin or ricin-like A chain may be prepared by expression of a truncated gene or by proteolytic degradation, for example with Nagarase (Funmatsu et al. , 1970, Jap. J. Med. Sci. Biol. 23:264-267). Similarly, the recombinant protein of the invention may contain only that portion of the B chain necessary for galactose recognition, cell binding and transport into the cell cytoplasm. Truncated B chains are described for example in EP 145,111. The A and B chains may be glycosylated or non-glycosylated. Glycosylated A and B chains may be obtained by expression in the appropriate host cell capable of glycosylation. Non-glycosylated chains may be obtained by expression in nonglycosylating host cells or by treatment to remove or destroy the carbohydrate moieties.

The proteins of the invention may be prepared using recombinant DNA methods. Accordingly, the nucleic acid molecules of the present invention may be incorporated in a known manner into an appropriate expression vector which ensures good expression of the protein. Possible expression vectors include but are not limited to cosmids, plasmids, or modified viruses (e.g. replication defective retroviruses, adenoviruses and adeno-associated viruses), so long as the vector is compatible with the host cell used. The expression vectors are "suitable for transformation of a host cell", means that the expression vectors contain a nucleic acid molecule of the invention and regulatory sequences selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid molecule. Operatively linked is intended to mean that the nucleic acid is linked to regulatory sequences in a manner which allows expression of the nucleic acid.

The invention therefore contemplates a recombinant expression vector of the invention containing a nucleic acid molecule of the invention, or a fragment thereof, and the necessary regulatory sequences for the transcription and translation of the inserted protein-sequence. Suitable regulatory sequences may be derived from a variety of sources,

including bacterial, fungal, viral, mammalian, or insect genes (For example, see the regulatory sequences described in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). Selection of appropriate regulatory sequences is dependent on the host cell chosen as discussed below, and may be readily accomplished by one of ordinary skill in the art. Examples of such regulatory sequences include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the host cell chosen and the vector employed, other sequences, such as an origin of replication, additional DNA restriction sites, enhancers, and sequences conferring inducibility of transcription may be incorporated into the expression vector. It will also be appreciated that the necessary regulatory sequences may be supplied by the native A and B chains and/or its flanking regions.

The recombinant expression vectors of the invention may also contain a selectable marker gene which facilitates the selection of host cells transformed or transfected with a recombinant molecule of the invention. Examples of selectable marker genes are genes encoding a protein such as G418 and hygromycin which confer resistance to certain drugs, β-galactosidase, chloramphenicol acetyltransferase, firefly luciferase, or an immunoglobulin or portion thereof such as the Fc portion of an immunoglobulin preferably IgG. Transcription of the selectable marker gene is monitored by changes in the concentration of the selectable marker protein such as β-galactosidase, chloramphenicol acetyltransferase, or firefly luciferase. If the selectable marker gene encodes a protein conferring antibiotic resistance such as neomycin resistance transformant cells can be selected with G418. Cells that have incorporated the selectable marker gene will survive, while the other cells die. This makes it possible to visualize and assay for expression of recombinant expression vectors of the invention and in particular to determine the effect of a mutation on expression and phenotype. It will be appreciated that selectable markers can be introduced on a separate vector from the nucleic acid of interest.

The recombinant expression vectors may also contain genes which encode a fusion moiety which provides increased expression of the recombinant protein; increased solubility of the recombinant protein; and aid in the purification of the target recombinant protein by acting as a ligand in affinity purification. For example, a proteolytic cleavage site may be added to the target recombinant protein to allow separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Typical fusion expression vectors include pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E

binding protein, or protein A, respectively, to the recombinant protein.

Recombinant expression vectors can be introduced into host cells to produce a transformant host cell. The term "transformant host cell" is intended to include prokaryotic and eukaryotic cells which have been transformed or transfected with a recombinant expression vector of the invention. The terms "transformed with", "transfected with", "transformation" and "transfection" are intended to encompass introduction of nucleic acid (e.g. a vector) into a cell by one of many possible techniques known in the art. Prokaryotic cells can be transformed with nucleic acid by, for example, electroporation or calcium-chloride mediated transformation. Nucleic acid can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofectin, electroporation or microinjection. Suitable methods for transforming and transfecting host cells can be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory textbooks.

Suitable host cells include a wide variety of prokaryotic and eukaryotic host cells. For example, the proteins of the invention may be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus), yeast cells or mammalian cells. Other suitable host cells can be found in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (199 1).

More particularly, bacterial host cells suitable for carrying out the present invention include *E. coli, B. subtilis, Salmonella typhimurium,* and various species within the genus' *Pseudomonas, Streptomyces,* and *Staphylococcus,* as well as many other bacterial species well known to one of ordinary skill in the art. Suitable bacterial expression vectors preferably comprise a promoter which functions in the host cell, one or more selectable phenotypic markers, and a bacterial origin of replication. Representative promoters include the β-lactamase (penicillinase) and lactose promoter system (see Chang et al., Nature 275:615, 1978), the trp promoter (Nichols and Yanofsky, Meth in Enzymology 101:155, 1983) and the tac promoter (Russell et al., Gene 20: 231, 1982). Representative selectable markers include various antibiotic resistance markers such as the kanamycin or ampicillin resistance genes. Suitable expression vectors include but are not limited to bacteriophages such as lambda derivatives or plasmids such as pBR322 (see Bolivar et al., Gene 2:9S, 1977), the pUC plasmids pUC18, pUC19, pUC118, pUC119 (see Messing, Meth in Enzymology 101:20-77, 1983 and Vieira and Messing, Gene 19:259-268, 1982), and pNH8A, pNH16a, pNH18a, and Bluescript M13 (Stratagene, La Jolla, Calif.). Typical fusion expression vectors which may be used are discussed above, e.g. pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ). Examples of inducible non-fusion expression vectors

include pTrc (Amann et al., (1988) Gene 69:301-315) and pET 11d (Studier et al., Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, California (1990) 60-89).

Yeast and fungi host cells suitable for carrying out the present invention include, but are not limited to *Saccharomyces cerevisae*, the genera *Pichia* or *Kluyveromyces* and various species of the genus *Aspergillus*. Examples of vectors for expression in yeast *S. cerivisae* include pYepSec1 (Baldari. et al., (1987) Embo J. 6:229-234), pMFa (Kurjan and Herskowitz, (1982) Cell 30:933-943), pJRY88 (Schultz et al., (1987) Gene 54:113-123), and pYES2 (Invitrogen Corporation, San Diego, CA). Protocols for the transformation of yeast and fungi are well known to those of ordinary skill in the art.(see Hinnen et al., PNAS USA 75:1929, 1978; Itoh et al., J. Bacteriology 153:163, 1983, and Cullen et al. (Bio/Technology 5:369, 1987).

Mammalian cells suitable for carrying out the present invention include, among others: COS (e.g., ATCC No. CRL 1650 or 1651), BHK (e.g., ATCC No. CRL 6281), CHO (ATCC No. CCL 61), HeLa (e.g., ATCC No. CCL 2), 293 (ATCC No. 1573) and NS-1 cells. Suitable expression vectors for directing expression in mammalian cells generally include a promoter (e.g., derived from viral material such as polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40), as well as other transcriptional and translational control sequences. Examples of mammalian expression vectors include pCDM8 (Seed, B., (1987) Nature 329:840) and pMT2PC (Kaufman et al. (1987), EMBOJ. 6:187-195).

Given the teachings provided herein, promoters, terminators, and methods for introducing expression vectors of an appropriate type into plant, avian, and insect cells may also be readily accomplished. For example, within one embodiment, the proteins of the invention may be expressed from plant cells (see Sinkar et al., J. Biosci (Bangalore) 11:47-58, 1987, which reviews the use of *Agrobacterium rhizogenes* vectors; see also Zambryski et al., Genetic Engineering, Principles and Methods, Hollaender and Setlow (eds.), Vol. VI, pp. 253-278, Plenum Press, New York, 1984, which describes the use of expression vectors for plant cells, including, among others, pAS2022, pAS2023, and pAS2034).

Insect cells suitable for carrying out the present invention include cells and cell lines from *Bombyx* or *Spodotera* species. Baculovirus vectors available for expression of proteins in cultured insect cells (SF 9 cells) include the pAc series (Smith et al., (1983) Mol. Cell Biol. 3:2156-2165) and the pVL series (Lucklow, V.A., and Summers, M.D., (1989) Virology 170:31-39). Some baculovirus-insect cell expression systems suitable for expression of the recombinant proteins of the invention are described in PCT/US/02442.

Alternatively, the proteins of the invention may also be expressed in non-human transgenic animals such as, rats, rabbits, sheep and pigs (see Hammer et al. (Nature

315:680-683, 1985), Palmiter et al. (Science 222:809-814, 1983), Brinster et al. (Proc Natl. Acad. Sci USA 82:44384442, 1985), Palmiter and Brinster (Cell. 41:343-345, 1985) and U.S. Patent No. 4,736,866).

The proteins of the invention may also be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, 1964, J. Am. Chem. Assoc. 85:2149-2154) or synthesis in homogenous solution (Houbenweyl, 1987, Methods of Organic Chemistry, ed. E. Wansch, Vol. 15 I and II, Thieme, Stuttgart).

The present invention also provides proteins comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for a retroviral protease, such as an HIV protease or an HTLV protease. Such a protein could be prepared other than by recombinant means, for example by chemical synthesis or by conjugation of A and B chains and a linker sequence isolated and purified from their natural plant, fungal or bacterial source. Such A and B chains could be prepared having the glycosylation pattern of the native ricin-like toxin.

N-terminal or C-terminal fusion proteins comprising the protein of the invention conjugated with other molecules, such as proteins may be prepared by fusing, through recombinant techniques. The resultant fusion proteins contain a protein of the invention fused to the selected protein or marker protein as described herein. The recombinant protein of the invention may also be conjugated to other proteins by known techniques. For example the proteins may be coupled using heterobifunctional thiol-containing linkers as described in WO 90/10457, N-succinimidyl-3-(2-pyridyldithio-proprionate) or N-succinimidyl-5-thioacetate. Examples of proteins which may be used to prepare fusion proteins or conjugates include cell binding proteins such as immunoglobulins, hormones, growth factors, lectins, insulin, low density lipoprotein, glucagon, endorphins, transferrin, bombesin, asialoglycoprotein glutathione-S-transferase (GST), hemagglutinin (HA), and truncated myc.

Utility of the Nucleic Acid Molecules and Proteins of the Invention

The proteins of the invention may be used to specifically inhibit or destroy mammalian cancer cells or mammalian cells infected with a retrovirus. It is an advantage of the recombinant proteins of the invention that they have specificity for the infected cells without the need for a cell binding component. The ricin-like B chain of the recombinant proteins recognize galactose moieties on the cell surface and ensure that the protein is taken up by the cell and released into the cytoplasm. When the protein is released into a non-infected cell, the A chain will remain inactive bound to the B chain. However, when the protein is released into a cell infected with a retrovirus or containing

an HTLV or HIV protease, the retroviral protease will cleave the cleavage recognition site in the linker, releasing the toxic A chain.

The specificity of a recombinant protein of the invention may be tested by treating the protein with the retroviral protease, such as HIV protease or HTLV protease which is thought to be specific for the cleavage recognition site of the linker and assaying for cleavage products. Retroviral proteases such as HIV protease or HTLV protease may be isolated from infected cells or may be prepared recombinantly, for example following the procedures in Darket et al. (1988, J. Biol. Chem. 254:2307-2312). The cleavage products may be identified for example based on size, antigenicity or activity. The toxicity of the recombinant protein may be investigated by subjecting the cleavage products to an in vitro translation assay in cell lysates, for example using Brome Mosaic Virus mRNA as a template. Toxicity of the cleavage products may be determined using a ribosomal inactivation assay (Westby et al. 1992, Bioconjugate Chem. 3:377-382). The effect of the cleavage products on protein synthesis may be measured in standardized assays of in vitro translation utilizing partially defined cell free systems composed for example of a reticulocyte lysate preparation as a source of ribosomes and various essential cofactors, such as mRNA template and amino acids. Use of radiolabelled amino acids in the mixture allows quantitation of incorporation of free amino acid precursors into trichloroacetic acid precipitable proteins. Rabbit reticulocyte lysates may be conveniently used (O'Hare, FEBS Lett. 1990, 273:200-204).

The ability of the recombinant proteins of the invention to selectively inhibit or destroy mammalian cells infected with a retrovirus such as cancer cells associated with HTLV or cells associated with HIV may be readily tested in vitro using mammalian cell cultures infected with the retrovirus of interest, or cancer lines. The selective inhibitory effect of the recombinant proteins of the invention may be determined by demonstrating the selective inhibition of viral antigen expression in mammalian cells, or selective inhibition of cellular proliferation in cancer cells or infected cells. For example, a selective inhibitory effect may be demonstrated by the selective inhibition of viral antigen expression in HIV-infected mononuclear phagocytic lineage cells; selective inhibition of cellular proliferation as measured against protein and DNA synthesis levels in treated, noninfected T cells and; selective loss of T cell viability. For example, the below-noted culture systems may be used to test the ability of recombinant proteins having a heterlogous linker sequence containing a cleavage recognition site for the HIV protease to selectively inhibit HIV infected cells. The term HIV refers to a CD4+ dependent human immunodeficiency retrovirus, such as HIV-1 and variants thereof.

Normal human T lymphocytes may be prepared from peripheral blood samples and cultured in vitro, as generally described in U.S. Patent No. 4,869,903. HIV infected

cells may also be obtained from AIDS patients. The cells may be infected in vitro with HIV derived from an AIDS patient. The toxicity of the recombinant protein for infected and non-infected cultures may then be compared. HIV-infected T cells express HIV envelope protein on the cell surface, in particular the proteins gp120 and gp41. The ability of the recombinant protein of the invention to inhibit the expression of these viral antigens may be an important indicator of the ability of the protein to inhibit viral replication. Toxicity may be measured based upon cell death or lysis, or by a reduction in the expression of HIV antigens, such as the major envelope proteins gp120 and gp41 or the HIV core protein antigen p24.

Levels of these antigens may be measured in assays using labelled antibodies having specificity for the antigens. Inhibition of viral antigen expression has been correlated with inhibition of viral replication (U.S. Patent No. 4,869,903). Similar assays may be carried out using other suitable mammalian cells which can be cultured in vitro and which are capable of maintaining retroviral replication. Examples of suitable cells include mononuclear phagocytic lineage cells. Toxicity may also be assessed based on a decrease in protein synthesis in target cells, which may be measured by known techniques, such as incorporation of labelled amino acids, such as [3H] leucine (O'Hare et al. 1990, FEBS Lett. 273:200-204). Infected cells may also be pulsed with radiolabelled thymidine and incorporation of the radioactive label into cellular DNA may be taken as a measure of cellular proliferation.

In the models of viral infection and replication for confirming the activity of the recombinant proteins of the invention, suitable mammalian cells used as hosts are those cells which can be cultured in vitro and which are capable of maintaining viral replication. Examples of suitable cells can be human T lymphocytes or mononuclear phagocytic lineage cells. Normal human T lymphocytes may be prepared from peripheral blood samples and cultured in vitro, as generally described in U.S. Patent No. 4,869,903. Virally infected cells may also be obtained from the blood of infected patients. The toxicity of the recombinant protein for infected and non-infected cultures may then be compared. The ability of the recombinant protein of the invention to inhibit the expression of these viral antigens may be an important indicator of the ability of the protein to inhibit viral replication. Levels of these antigens may be measured in assays using labelled antibodies having specificity for the antigens. Inhibition of viral antigen expression has been correlated with inhibition of viral replication (U.S. Patent No. 4,869,903).

Toxicity may also be assessed based on a decrease in protein synthesis in target cells, which may be measured by known techniques, such as incorporation of labelled amino acids, such as [3H] leucine (O'Hare et al. 1990, FEBS Lett. 273:200-204). Infected

cells may also be pulsed with radiolabelled thymidine and incorporation of the radioactive label into cellular DNA may be taken as a measure of cellular proliferation. In addition, toxicity may be measured based on cell viability, for example the viability of infected and non-infected cell cultures exposed to the recombinant protein may be compared. Cell viability may be assessed by known techniques, such as trypan blue exclusion assays.

Although, the specificity of the proteins of the invention for retrovirally infected cells is mediated by the specific cleavage of the cleavage recognition site of the linker, it will be appreciated that specific cell binding components may optionally be conjugated to the proteins of the invention. Such cell binding components may be expressed as fusion proteins with the proteins of the invention or the cell binding component may be physically or chemically coupled to the protein component. Examples of suitable cell binding components include antibodies to retroviral proteins, or to cancer cell proteins.

Antibodies having specificity for a cell surface protein may be prepared by conventional methods. A mammal, (e.g., a mouse, hamster, or rabbit) can be immunized with an immunogenic form of the peptide which elicits an antibody response in the mammal. Techniques for conferring immunogenicity on a peptide include conjugation to carriers or other techniques well known in the art. For example, the peptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay procedures can be used with the immunogen as antigen to assess the levels of antibodies. Following immunization, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera.

To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art, (e.g., the hybridoma technique originally developed by Kohler and Milstein (Nature 256, 495-497 (1975)) as well as other techniques such as the human B-cell hybridoma technique (Kozbor et al., Immunol. Today 4, 72 (1983)), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al. Monoclonal Antibodies in Cancer Therapy (1985) Allen R. Bliss, Inc., pages 77-96), and screening of combinatorial antibody libraries (Huse et al., Science 246, 1275 (1989)]. Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with the peptide and the monoclonal antibodies can be isolated.

The term "antibody" as used herein is intended to include fragments thereof which also specifically react with a cell surface component. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as

described above. For example, F(ab')2 fragments can be generated by treating antibody with pepsin. The resulting F(ab')2 fragment can be treated to reduce disulfide bridges to produce Fab' fragments.

Chimeric antibody derivatives, i.e., antibody molecules that combine a non-human animal variable region and a human constant region are also contemplated within the scope of the invention. Chimeric antibody molecules can include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species, with human constant regions. Conventional methods may be used to make chimeric antibodies containing the immunoglobulin variable region which recognizes a cell surface antigen (See, for example, Morrison et al., Proc. Natl Acad. Sci. U.S.A. 81,6851 (1985); Takeda et al., Nature 314, 452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., European Patent Publication EP171496; European Patent Publication 0173494, United Kingdom patent GB 2177096B). It is expected that chimeric antibodies would be less immunogenic in a human subject than the corresponding non-chimeric antibody.

Monoclonal or chimeric antibodies specifically reactive against cell surface components can be further humanized by producing human constant region chimeras, in which parts of the variable regions, particularly the conserved framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin. Such immunoglobulin molecules may be made by techniques known in the art, (e.g., Teng et al., Proc. Natl. Acad. Sci. U.S.A., 80, 7308-7312 (1983); Kozbor et al., Immunology Today, 4, 7279 (1983); Olsson et al., Meth. Enzymol., 92, 3-16 (1982)), and PCT Publication WO92/06193 or EP 0239400). Humanized antibodies can also be commercially produced (Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great Britain.)

Specific antibodies, or antibody fragments, reactive against cell surface components may also be generated by screening expression libraries encoding immunoglobulin genes, or portions thereof, expressed in bacteria with cell surface components. For example, complete Fab fragments, VH regions and FV regions can be expressed in bacteria using phage expression libraries (See for example Ward et al., Nature 341, 544-546: (1989); Huse et al., Science 246, 1275-1281 (1989); and McCafferty et al. Nature 348, 552-554 (1990)). Alternatively, a SCID-hu mouse, for example the model developed by Genpharm, can be used to produce antibodies, or fragments thereof.

The proteins of the invention may be formulated into pharmaceutical compositions for adminstration to subjects in a biologically compatible form suitable for administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the substance to be administered in which any toxic effects are

outweighed by the therapeutic effects. The substances may be administered to living organisms including humans, and animals. Administration of a therapeutically active amount of the pharmaceutical compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a substance may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of antibody to elicit a desired response in the individual. Dosage regime may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The active substance may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active substance may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound.

The compositions described herein can be prepared by per se known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985). On this basis, the compositions include, albeit not exclusively, solutions of the substances in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

The pharmaceutical compositions may be used in methods for treating mammals, including humans, infected with a retrovirus. It is anticipated that the compositions will be particularly useful for treating patients infected with HIV-1, HIV-2 or cancers involving retroviruses, such as human T-cell leukemias involving HTLV. The efficacy of such treatments may be monitored by assessing the health of the patient treated and by measuring the percentage of HIV positive monocytes in treated patients.

The dose of the recombinant protein to be administered will depend on a variety of factors which may be readily monitored in human subjects. Such factors include HIV antigen levels associated with HIV infected T cells or mononuclear phagocytes; HIV antigen levels in the bloodstream; reverse transcriptase activity associated with HIV-infected T cells or mononuclear phagocytes; and the ratio of viable HIV infected cells to uninfected cells. HIV antigen levels in plasma, for example, may be readily determined using an ELISA assay.

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

EXAMPLE 1

Cloning and Expression of Proricin Variants Activated by HIV Proteases

Isolation of total RNA

The preproricin gene was cloned from new foliage of the castor bean plant. Total messenger RNA was isolated according to established procedures (Maniatis et al., *Molecular Cloning: A Lab Manual* (Cold Spring Harbour Press, Cold Spring Harbour, (1989)) and cDNA generated using reverse transcriptase.

cDNA Synthesis:

Oligonucleotides, corresponding to the extreme 5' and 3' ends of the preproricin gene were synthesized and used to PCR amplify the gene. Using the cDNA sequence for preproricin (Lamb et al., Eur. J. Biochem., 145:266-270, 1985), several oligonucleotide primers were designed to flank the start and stop codons of the preproricin open reading frame. The oligonucleotides were synthesized using an Applied Biosystems Model 392 DNA/RNA Synthesizer. First strand cDNA synthesis was primed using the oligonucleotide Ricin1729C (Table 1). Three micrograms of total RNA was used as a template for oligo Ricin1729C primed synthesis of cDNA using Superscript II Reverse Transcriptase (BRL) following the manufacturer's protocol.

DNA Amplification and Cloning

The first strand cDNA synthesis reaction was used as template for DNA amplification by the polymerase chain reaction (PCR). The preproricin cDNA was amplified using the upstream primer Ricin-99 and the downstream primer Ricin1729C with Vent DNA polymerase (New England Biolabs) using standard procedures (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)). Amplification was carried out in a Biometra thermal cycler (TRIO-Thermalcycler) using the following cycling parameters: denaturation 95°C for 1 min., annealing 52°C for 1 min., and extension 72°C for 2 min., (33 cycles), followed by a final extension cycle at 72°C for 10 min. The 1846bp amplified product was fractionated on an agarose gel (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989), and the DNA purified from the gel slice using Qiaex resin (Qiagen) following the manufacturer's protocol. The purified PCR fragment encoding the preproricin cDNA was then ligated (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)) into an Eco RV-digested pBluescript II SK plasmid (Stratagene), and used to transform competent XL1-Blue cells (Stratagene). Positive clones were confirmed by restriction digestion of purified plasmid DNA. Plasmid DNA was extracted using a

Qiaprep Spin Plasmid Miniprep Kit (Qiagen).

DNA Sequencing

The cloned PCR product containing the putative preproricin gene was confirmed by DNA sequencing of the entire cDNA clone (pAP-144). Sequencing was performed using an Applied Biosystems 373A Automated DNA Sequencer, and confirmed by double-stranded dideoxy sequencing by the Sanger method using the Sequenase kit (USB). The oligonucleotide primers used for sequencing were as follows: Ricin267, Ricin486, Ricin725, Ricin937, Ricin1151, Ricini1399, Ricin1627, T3 primer (5'AATTAACCCTCACTAAAGGG-3') and T7 primer (5'GTAATACGACTCACTATAGGGC-3). Sequence data was compiled and analyzed using PC Gene software package (intelligenetics). The sequences and location of oligonucleotide primers is shown in Table 1.

Mutagenesis of Preproricin Linker

The preproricin cDNA clone (pAP-144) was subjected to site-directed mutagenesis in order to generate a series of variants differing only in the sequence between the A and B chains (linker region). The wild-type preproricin linker region was replaced with the three linker sequences, pAP-146, pAP-147 and pAP-148 displayed in Figure 4. The linker regions of the variants encode an HIV protease cleavage recognition sequence (Slalka et al., Cell, 56:911-913, 1989). The mutagenesis and cloning strategy used to generate the linker variant pAP-146 is summarized in Figures 1A and 1B. The mutagenesis and cloning strategy used to generate the linker variant pAP-147 is summarized in Figures 2A and 2B. The mutagenesis and cloning strategy used to generate the linker variant pAP-148 is summarized in Figures 3A and 3B. The first step involved a DNA amplification using a set of mutagenic primers (HIVA1; HIVB1; HIVH1) in combination with the two flanking primers Ricin-99Eco and Ricin1729Xba. The PCR protocol and conditions used were the same as described above. PCR products from each mutagenesis reaction were gel purified then restriction digested with either Eco R1 for the A-chain encoding fragment, or Xba I for the B chain encoding fragment. Restriction digested PCR fragments were gel purified and then ligated with PBluescript SK which had been digested with Eco RI and Xba I. Ligation reactions were used to transform competent XL1-Blue cells (Stratagene). Recombinant clones were identified by restriction digests of plasmid miniprep DNA and the mutant linker sequence were confirmed by DNA sequencing.

Subcloning Preproricin Mutants into Vector pSB2

Full length preproricin cDNA was created from clones pAP-146, pAP-147, and pAP-148, which lack the first three nucleotides of the signal sequence (Halling et al, Nucleic Acids Research, 13:8019-8033, 1985). The missing ATG (start codon) was introduced into each mutant by site-directed mutagenesis using primers Ricin-109 and

Ricin1729C. The DNA template for each reaction was pAP-146, pAP-147, or pAP-148, and the PCR conditions were the same as described above. PCR products were gel purified and then ligated with Sma I-digested pSB2 (see Figure 12). Recombinant clones were identified by restriction digests of plasmid miniprep DNA, and the 5′ and 3′ junctions confirmed by DNA sequencing. The three constructs obtained were pAP-151, pAP-159, and pAP-163, with each having the mutant linker found in pAP-146, pAP-147, and pAP-148 respectively.

Subcloning Preproricin Mutants into Vector pVL1393

Preproricin variants were subcloned into the baculovirus transfer vector pVL1393 (PharMingen, sequence shown in Figure 11). The subcloning strategy for the HIV-A linker variant is summarized in Figure 5. The subcloning strategy for the HIV-B linker variant is summarized in Figure 6. The subcloning strategy for the HIV-H linker variant is summarized in Figure 7. The 1315 bp Eco RI/Kpn I fragment encoding the ricin A-chain and each mutant linker was isolated from each of the variant clones in pSB2 (pAP-151, pAP-159, and pAP-163). Each of these purified fragments was ligated with a 564 bp KpnI/PStI fragment obtained from pAP-144, and with Eco RI/Pst I cleaved pVL1393. Recombinant clones were identified by restriction digests of plasmid miniprep DNA and the 5′ and 3′ junctions confirmed by DNA sequencing. The three constructs obtained were pAP-190, pAP-196, and pAP-197, each having the mutant linker found in pAP-146, pAP-147, and pAP-148, respectively.

Isolation of Recombinant Baculoviruses

Insect cells *S. frugiperda* (Sf9), and *Trichoplusia ni* (Tn368 and BTI-TN-581-4 (High Five)) were maintained on TMN-FH medium supplemented with 10% total calf serum (Summers et al., A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987)). Two micrograms of recombinant pVL1393 DNA (pAP-190, pAP-196, or pAP-197) was co-transfected with 0.5 microgram of BaculoGold AcNPV DNA (Pharmingen) into 2 x 10^6 Tn368 insect cells following the manufacturer's protocol (Gruenwald et al., Baculovirus Expression Vector System: Procedures and Methods Manual, 2nd Edition, (San Diego, CA, 1993)). On day 5 post-transfection, media were centrifuged and the supernatants tested in limiting dilution assays with Tn368 cells (Summers et al., A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987)). Recombinant viruses in the supernatants were then amplified by infecting Tn368 cells at a multiplicity of infection (moi) of 0.1, followed by collection of day 7 supernatants. A total of three rounds of amplification were performed for each recombinant following established procedures (Summers et al., A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987 and

Gruenwald et al., Baculovirus Expression Vector System: Procedures and Methods Manual, 2nd Edition, (San Diego, CA, 1993)).

Expression of Mutant Proricin

Recombinant baculoviruses (pAP-190baculo, pAP-196baculo, and pAP-197-baculo) were used to infect 2 x 10^5 Tn368 or sf9 cells of an moi of 5 in EX-CELL400 media (JRH Biosciences) with 25mM α-lactose in spinner flasks. Media supernatants containing mutant proricins were collected on day 6 post-infection.

Purification of Mutant Proricin

Media supernatants were ultracentrifuged at 100,000g for 1 hour. After the addition of 1 mM phenylmethylsulfonyl fluoride, the supernatants were concentrated using an Amicon 8050 Ultrafiltration Cell fitted with a Diaflo XM50 membrane. Supernatants were then dialysed extensively against 137 mN NaCl, 2.2 mM KCl, 2.6 mM KH_2PO_4, and 8.6 mM Na_2HPO_4, pH 7.4 containing 1 mM dithiothreitol (dialysis buffer). Recombinant proricin proteins were purified by affinity chromatography using lactose agarose (Sigma) as previously described for recombinant ricin-B chain (Ferrini et al., Eur. J. Biochem., 233:772-777, 1995). Fractions containing recombinant proricin were identified using SDS/PAGE, (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989) and by Western blot analysis using anti-ricin antibodies (Sigma).

In Vitro HIV Protease Digestion of Proricin Variants

Affinity -purified mutant proricin was treated with HIV protease to confirm specific cleavage in the linker region. Proricin variants were eluted from the lactose-agarose matrix in protease digestion buffer (50mM NaCl, 50mM Na-acetate, pH 5.5, 1mM dithiothreitol) containing 100mM lactose. Proricin substrate was then incubated at 37°C for 60 minutes with 400 ng/ml recombinant HIV protease (BACHEM Biosciences Inc.). The cleavage products of proricin (ricin A and B chains) were identified using SDS/PAGE (Sambrook et al., *Molecular Cloning: a Laboratory Manual*, 2nd. ed., Cold Spring Harbor Press, 1989), followed by Western blot analysis using anti-ricin antibodies (Sigma).

In Vitro Translation Assay

The activity of protease-treated proricin variants was monitored using a rabbit reticulocyte lysate in a non-radioactive (Amersham, ECL system) *in vitro* translation assay. Protease-treated proricin was added to a standard 50 µl translation reaction mix containing Brome Mosaic Virus mRNA as template (following the manufacturer's protocol). Active ricin variants inhibit the *in vitro* translation reaction by inactivating ribosomes. Therefore, in the presence of an active ricin variant, no viral proteins are synthesized.

EXAMPLE 2

Harvesting and affinity column purification of pro-ricin variants

Protein samples were harvested three days post transfection. The cells were removed by centrifuging the media at 1465 g for ten minutes using a SLA-1500 (Sorvall) centrifuge rotor. The supernatant was further clarified by centrifuging at 7970g for fifteen minutes. Protease inhibitor phenylmethyl-sulfonyl fluoride (Sigma) was added to a final concentration of 1%. The samples were concentrated (five-fold) and dialyzed (four times five-fold) into dialysis buffer (1X baculo buffer (8.6 mM Na_2HPO_4, 2.6 mM KH_2PO_4, 137 mM naCl and 2.6 mM KCl, pH 7.4) containing 2.5 mM lactose, and 0.02% NaN_3) using a MINITAN concentrator (Millipore) with 30kDa NMWL plates. Dithiothreitol (DTT) was then added to a final concentration of 1 mM, and the samples were centrifuged at 37000g for one hour.

Following centrifugation, dialysis buffer containing 1 mM DTT was added to the samples to a final volume of 500 mL. The samples were degassed and applied overnight at 4°C to an ASF-sepharose affinity column (prepared according to Pharmacia protocol) in a 10 mL chromatography column (Biorad). The column was washed with 300 mL of wash buffer (100 mM NaOAc, pH 5.2, 1mM DTT, and 0.02% NaN_3). Elution of pro-ricin variant was performed by applying 500 mL of elution buffer (100 mM NaOAc, pH 5.2, 250 mM lactose, and 5 mM DTT). The eluate was concentrated using an Amicon 8050 concentrator (Amicon) with a YM10 176 mm membrane, utilizing argon gas to pressurize the chamber. The samples were further concentrated and dialyzed into 1X Baculo buffer using Ultrafree-15 Biomax (Millipore) 10 kDa NMWL filter devices, which were spun in a Beckman S4180 rotor (Beckman) at 2000g. Samples were flash frozen in dry ice and stored at -20°C.

Purification of pAP 190 by gel filtration chromatography

In order to purify the pro-ricin variant from processed material produced during fermentation, the protein was applied to a SUPERDEX 75 (16/60) column and SUPERDEX 200 (16/60) column (Pharmacia) connected in series equilibrated with 50 mM Tris, 100mM NaCl, pH 7.5 containing 100 mM Lactose and 0.1% β-mercaptoethanol (βME). The flow rate of the column was 0.15 mL/min and fractions were collected every 25 minutes. The UV (280 nm) trace was used to determine the approximate location of the purified pAP 190 and thus determine the samples for Western analysis.

Western analysis of column fractions

Fractions eluted from the SUPERDEX columns (Pharmacia) were analyzed for purity using standard Western blotting techniques. An aliquot of 10μL from each fraction was boiled in 1X sample buffer (62.6 mM Tris-Cl, pH 6.8, 4.4% βME, 2% sodium dodecyl sulfate (SDS), 5% glycerol (all from Sigma) and 0.002% bromophenol blue (Biorad)) for

five minutes. Denatured samples were loaded on 12% Tris-Glycine Gels (Biorad) along with 50 ng of RCA_{60} (Sigma) and 5 µL of kaleidoscope prestained standards (Biorad). Electrophoresis was carried out for ninety minutes at 100V in 25 mM Tris-Cl, pH 8.3, 0.1% SDS, and 192 mM glycine using the BioRad Mini Protean II cells (Biorad).

Following electrophoresis gels were equilibrated in transfer buffer (48 mM Tris, 39 mM glycine, 0.0375% SDS, and 20% Methanol) for a few minutes. PVDF Biorad membrane was presoaked for one minute in 100% methanol and two minutes in transfer buffer. Whatman paper was soaked briefly in transfer buffer. Five pieces of Whatman paper, membrane, gel, and another five pieces of Whatman paper were arranged on the bottom cathode (anode) of the Pharmacia Novablot transfer apparatus (Pharmacia). Transfer was for one hour at constant current (2 mA/cm^2).

Transfer was confirmed by checking for the appearance of the prestained standards on the membrane. Non-specific sites on the membrane were blocked by incubating the blot for thirty minutes in 1X Phosphate Buffered Saline (1X PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) with 5% skim milk powder (Carnation). Primary antibody (Rabbit α-ricin, Sigma) was diluted 1:3000 in 1X PBS containing 0.1% Tween 20 (Sigma) and 2.5% skim milk and incubated with blot for forty five minutes on a orbital shaker (VWR). Non-specifically bound primary antibody was removed by washing the blot for ten minutes with 1X PBS containing 0.2% Tween 20. This was repeated four times. Secondary antibody donkey anti-rabbit (Amersham) was incubated with the blot under the same conditions as the primary antibody. Excess secondary antibody was washed as described above. Blots were developed with the ECL Western Blotting detection reagents according to the manufacturer's instructions. Blots were exposed to Medtec's Full Speed Blue Film (Medtee) or Amersham's ECL Hyperfilm (Amersham) for three to fifteen minutes. Film was developed in a KODAK Automatic Developer.

Determination of lectin binding ability of pro-ricin variant

An Immulon 2 place (VDVR) was coated with 100 µl per well of 10µg/ml of asialofetuin and left overnight at 4°C. The plate was washed with 3X 300 µL per well with ddH_2O using an automated plate washer (BioRad). The plate was blocked for one hour at 37°C by adding 300 µL per well of PBS containing 1% ovalbumin. The plate was washed again as above. Pro-ricin variant pAP 190 was added to the plate in various dilutions in 1X Baculo. A standard curve of RCA_{60} (Sigma) from 1-10 ng was also included. The plate was incubated for 1 h at 37°C. The plate was washed as above. Anti-ricin monoclonal antibody (Sigma) was diluted 1:3000 in 1X PBS containing 0.5% ovalbumin and 0.1% tween-20, added at 100 µL per well and incubated for 1 h at 37°C. The plate was

washed as above. Donkey-anti rabbity polyclonal antibody was diluted 1:3000 in 1X PBS containing 0.5% ovalbumin, 0.1% Tween-20, and added at 100µL per well and incubated for 1 h at 37°C. The plate was given a final wash as described above. Substrate was added to plate at 100µL per well (1 mg/ml o-phenylenediamine (Sigma), 1 µL/ml H_2O_2, 25 µL of stop solution (20% H_2SO_4) was added and the absorbance read (A490nm-A630nm) using a SPECTRA MAX 340 plate reader (Molecular Devices).

Determination of pAP 190 activity using the rabbit reticulocyte assay

Ricin sample were prepared for reduction.

A) RCA_{60} = 3,500 ng/µL of RCA_{60} + 997 µL 1xEndo buffer (25mM Tris, 25mM KCl,5mM $MGCl_2$, pH 7.6)

Reduction = 95 µL of 10ng/µL + 5 µL β-mercaptoethanol

B) Ricin variants

Reduction = 40 µL variant + 2 µL β-mercaptoethanol

The ricin standard and the variants were incubated for 30 minutes at room temperature.

Ricin - Rabbit Reticulocyte lysate reaction

The required number of 0.5 mL tubes were labelled. (2 tubes for each sample, + and - animline). To each of the sample tubes 20 µL of 1X endo buffer was added, and 30 µL of buffer was added to the controls. To the sample tubes either 10 µL of 10ng/µL Ricin or 10µL of variant was added. Finally, 30µL of rabbit reticulocyte lysate was added to all the tubes. The samples were incubated for 30 minutes at 30°C using the thermal block. Samples were removed from the eppendorf tube and contents added into a 1.5 mL tube containing 1 mL of TRIZOL (Gibco). Samples were incubated for 15 minutes at room temperature. After the incubation, 200 µL of chloroform was added, and the sample was vortexed and spun at 12,000 g for 15 minutes at 4°C. The top aqueous layer from the samples was removed and contents added to a 1 mL tube containing 500 µL of isopropanol. Samples were incubated for 15 minutes at room temperature and then centrifuged at 12,000 for 15 minutes at 4°C. Supernatant was removed and the pellets were washed with 1 mL of 70% ethanol. Centrifugation at 12,000 g for 5 minutes at 4°C precipitated the RNA. All but approximately 20 µL of the supernatant was removed and the remaining liquid evaporated using the speed vacuum machine. The control samples (-aniline) were dissolved in 10 µL of 0.1 X E buffer (36 mM Tris, 30 mM NaH_2PO_4, 1 mM EDTA, pH 7.8) and stored at -70°C or on dry ice until later. Pellets from the other samples (+aniline samples) were dissolved in 20 µL of DEPC treated ddH_2O. An 80 µL aliquot of 1 M aniline (distilled) with 2.8 M acetic acid was added to these RNA samples and transferred to a fresh 0.5 mL tube. The samples were incubated in the dark for 3 minutes at 60°C. RNA

was precipitated by adding 100 µL of 95% ethanol and 5µL of 3M sodium acetate, pH 5.2 to each tube and centrifuging at 12,000 g for 30 minutes at 4°C. Pellets were washed with 1 mL 70% ethanol and centrifuged again at 12,000g for 5 minutes at 4°C to precipitate RNA. The supernatant was removed and excess liquid evaporated using the speed vacuum machine. These pellets (+ aniline samples) were dissolved in 10µL of 0.1 X E buffer. To all samples (+ and - aniline), 10 µL of formamide loading dye was added. The RNA ladder (8 µL of ladder + 8 µL of loading dye) was also included. Samples were incubated for 2 minutes at 70°C on the thermal block. Electrophoresis was carried out on the samples using 1.2% agarose, 50% formamide gels in 0.1X E buffer + 0.2% SDS. The gel was run for 90 minutes at 75 watts. RNA was visualized by staining the gel in 1 µg/µL ethidium bromide in running buffer for 45 minutes. The gel was examined on a 302 nm UV box and photographed using the gel documentation system.

Results:

Protein Expression Yields

Aliquots were taken at each stop of the harvesting/purification and tested. Yields of functional ricin variant were determined by ELISA. Typical results on an 800 mL prep of infected *T. ni* cells are given below.

Aliquot	µg pAP 190
Before concentration and dialysis	648.5
After concentration and dialysis	364.4
ASF column flow through	62.1
ASF column elution	300.7

Yield: 300.7/648.5 = 46.4%

Purification of pAP 190 and Western Analysis of column fractions

Partially purfied pAP 190 was applied to Superdex 75 and 200 (16/60) columns connected in series in order to remove the contaminating non-specifically processed pAP 190. Eluted fractions were tested via Western analysis and the fractions containing the most pure protein were pooled, concentrated and re-applied to the column. The variant was applied a total of three times to the column. The final purified pAP 190 has less than 1% processed variant. Figure 13 shows that the purified pAP 190 is in three fractions and the processed material eluted in two separate fractions.

The purified pAP 190 was tested for susceptibility to cleavage by HIV protease and for activation of the A-chain of the pro-ricin variant, (inhibition of protein

synthesis). PAP 190 was incubated with and without HIV protease for a specified time period and then electrophoresed and blotted. Cleaved pAP 190 will run as two 30 kDa proteins (B is slightly larger) under reducing (SDS-PAGE) conditions. Unprocessed pAP 190, which contains the linker region, will run at 60 kDa. HIV protease was able to cleave the pAP 190 (shown in Figure 14). Lanes B and D show untreated; while lanes C and E to G show HIV protease treated pAP 190.

Activation of pAP 190 variant with HIV protease

Activation of HIV protease treated pAP 190 190, based on the method of *May et al.* (EMBO Journal. 8 301-8, 1989) was demonstrated in Figure 15. The appearance of the 390 based pair product is observed in lane B, which is the positive control, and not observed in lane C, the negative control. Lanes D-G show that there was no N-glycosidase activity in the pAP 190 variant as predicted. Lanes H-K show that processed pAP 190 possesses N-glycosidase activity as predicted.

The pAP 190 variant has been expressed in insect cells, purified to greater than 99%, and activation of the variant has been demonstrated by cleavage with HIV protease.

Example 3

Cloning and Expression of Proricin Variants Activated by HTLV

Isolation of total RNA:

The preproricin gene is cloned from new foliage of the castor bean plant. Total messenger RNA is isolated according to established procedures (Maniatis et al., Molecular Cloning: A Lab Manual (Cold Spring Harbour Press, Cold Spring Harbour, (1989)) and cDNA generated using reverse transcriptase.

cDNA Synthesis:

Oligonucleotides, corresponding to the extreme 5' and 3' ends of the preproricin gene are synthesized and used to PCR amplify the gene. Using the cDNA sequence for preproricin (Lamb et al., *Eur. J. Biochem.* 145:266-270 (1985)), several oligonucleotide primers are designed to flank the start and stop codons of the preproricin open reading frame. The oligonucleotides are synthesized using an Applied Biosystems Model 392 DNA/RNA Synthesizer. First strand cDNA synthesis is primed using the oligonucleotide Ricin1729C (Table 1). Three micrograms of total RNA is used as a template for oligo Ricin1729C primed synthesis of cDNA using Superscript II Reverse Transcriptase (BRL) following the manufacturer's protocol.

DNA Amplification and Cloning:

The first strand cDNA synthesis reaction is used as template for DNA amplification by the polymerase chain reaction (PCR). The preproricin cDNA is amplified using the upstream primer Ricin-109 and the downstream primer Ricin1729C

with Vent DNA polymerase (New England Biolabs) using standard procedures (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)). Amplification is carried out in a Biometra thermal cycler (TRIO-Thermalcycler) using the following cycling parameters: denaturation 95°C for 1 min., annealing 52°C for 1 min., and extension 72°C for 2 min., (33 cycles), followed by a final extension cycle at 72°C for 10 min. The 1846bp amplified product is fractionated on an agarose gel (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989), and the DNA purified from the gel slice using Qiaex resin (Qiagen) following the manufacturer's protocol. The purified PCR fragment encoding the preproricin cDNA is then ligated (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989)) into an Eco RI-digested pBluescript II SK plasmid (Stratagene), and used to transform competent XL1-Blue cells (Stratagene). Positive clones are confirmed by restriction digestion of purified plasmid DNA. Plasmid DNA is extracted using a Qiaprep Spin Plasmid Miniprep Kit (Qiagen).

DNA Sequencing:

The cloned PCR product containing the putative preproricin gene is confirmed by DNA sequencing of the entire cDNA clone (pAP-144). Sequencing is performed using an Applied Biosystems 373A Automated DNA Sequencer, and confirmed by double-stranded dideoxy sequencing by the Sanger method using the Sequenase kit (USB). The oligonucleotide primers used for sequencing are as follows: Ricin267, Ricin486, Ricin725, Ricin937, Ricin1151, Ricin 1399, Ricin1627, T3 primer (5'AATTAACCCTCACTAAAGGG-3') and T7 primer (5'GTAATACGACTCACTATAGGGC-3). Sequence data is compiled and analyzed using PC Gene software package (intelligenetics). The sequences and location of oligonucleotide primers is shown in Table 1.

Mutagenesis of Preproricin Linker:

The preproricin cDNA clone (pAP-144) is subjected to site directed mutagenesis in order to generate a series of variants differing only in the sequence between the A and B chains (linker region). The wild-type preproricin linker region is replaced with the linker sequences displayed in Figure 20. The linker regions of the variants encode a disease-specific protease cleavage recognition sequence (Slalka et al., *Cell*, 56:911-913, 1989). The mutagenesis and cloning strategy used to generate the HTLV protease-sensitive linker variants is summarized in Figures 16A, 17A, 18A and 19A.

The first step involves a DNA amplification using a set of mutagenic primers encoding for the disease-specific protease-sensitive linker in combination with the two flanking primers Ricin-109Eco and Ricin1729Xba. The PCR protocol and conditions used

are the same as described above. PCR products from each mutagenesis reaction are gel purified then restriction digested with either Eco R1 for the A-chain encoding fragment, or Xba I for the B chain encoding fragment. Restriction digested PCR fragments are gel purified and then ligated with pBluescript SK which has been digested with Eco RI and Xba I. Ligation reactions are used to transform competent XL1-Blue cells (Stratagene). Recombinant clones are identified by restriction digests of plasmid miniprep DNA and the mutant linker sequence are confirmed by DNA sequencing.

Subcloning Preproricin Mutants into Vector pVL1393:

Preproricin variants are subcloned into the baculovirus transfer vector pVL1393 (PharMingen). The subcloning strategy for the HTLV protease-sensitive linker variants is summarized in Figures 16C, 17C, 18C, and 19C. The 1315 bp Eco RI/Kpn I fragment encoding the ricin A-chain and each mutant linker is isolated from pAP-205, pAP-207, pAP-209 or pAP-211. Each of these purified fragments is ligated with a 564 bp KpnI/PStI fragment obtained from pAP-144, and with Eco RI/Pst I cleaved pVL1393. Recombinant clones are identified by restriction digests of plasmid miniprep DNA and the 5' and 3' junctions confirmed by DNA sequencing.

Isolation of Recombinant Baculoviruses:

Insect cells *S. frugiperda* (Sf9), and *Trichoplusia ni* (Tn368 and BTI TN-581-4 (High Five)) are maintained on TMN-FH medium supplemented with 10% total calf serum (Summers et al., A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987)). Two micrograms of recombinant pVL1393 DNA (pAP-190, pAP-196, or pAP-197) is co-transfected with 0.5 microgram of BaculoGold AcNPV DNA (Pharmingen) into 2 x 106 Tn368 insect cells following the manufacturer's protocol (Gruenwald et al., Baculovirus Expression Vector System: Procedures and Methods Manual, 2nd Edition, (San Diego, CA, 1993)). On day 5 post-transfection, media are centrifuged and the supernatants tested in limiting dilution assays with Tn368 cells (Summers et al., A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987)). Recombinant viruses in the supernatants are then amplified by infecting Tn368 cells at a multiplicity of infection (moi) of 0.1, followed by collection of day 7 supernatants. A total of three rounds of amplification are performed for each recombinant following established procedures (Summers et al., A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures, (Texas Agricultural Experiment Station, 1987 and Gruenwald et al., Baculovirus Expression Vector System: Procedures and Methods Manual, 2nd Edition, (San Diego, CA, 1993)).

Expression of Mutant Proricin:

Recombinant baculoviruses (pAP-206-baculo, pAP-208-baculo, pAP-210-baculo, and pAP-212-baculo) are used to infect 2 x 105 Tn368 or sf9 cells of an moi of 5 in EX-CELL400 media (JRH Biosciences) with 25mM a-lactose in spinner flasks. Media supernatants containing mutant proricins are collected on day 6 post-infection.

Purification of Mutant Proricin:

Media supernatants are ultracentrifuged at 100,000g for 1 hour. After the addition of 1 mM phenylmethylsulfonyl fluoride, the supernatants are concentrated using an Amicon 8050 Ultrafiltration Cell fitted with a Diaflo XM50 membrane. Supernatants are then dialysed extensively against 137 mN NaCl, 2.2 mM KCl, 2.6 mM KH_2PO_4, and 8.6 mM Na_2HPO_4, pH 7.4 containing 1 mM dithiothreitol (dialysis buffer). Recombinant proricin proteins are purified by affinity chromatography using lactose agarose (Sigma) as previously described for recombinant ricin-B chain (Ferrini et al., *Eur. J. Biochem.* 233:772-777 (1995)). Fractions containing recombinant proricin are identified using SDS/PAGE, (Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, (Cold Spring Harbor Laboratory Press, 1989) and by Western blot analysis using anti-ricin antibodies (Sigma).

In Vitro Protease Digestion of Proricin Variants:

Affinity-purified proricin variant is treated with individual disease-specific proteases to confirm specific cleavage in the linker region. Ricin-like toxin variants are eluted from the lactose-agarose matrix in protease digestion buffer (50mM NaCl, 50mM Na-acetate, pH 5.5, 1mM dithiothreitol) containing 100mM lactose. Proricin substrate is then incubated at 37°C for 60 minutes with a disease-specific protease. The cleavage products consisting ricin A and B chains are identified using SDS/PAGE (Sambrook et al., Molecular Cloning: a Laboratory Manual, 2nd. ed., Cold Spring Harbor Press, 1989), followed by Western blot analysis using anti-ricin antibodies (Sigma).

HTLV proteases may be obtained from Bachem Bioscience. Cathepsin B may be obtained from Medcor or Calbiochem.

In Vitro Translation Assay:

The activity of protease-treated ricin-like toxin variants is monitored using a rabbit reticulocyte lysate in a non-radioactive (Amersham, ECL system) *in vitro* translation assay. Protease-treated proricin is added to a standard 50 ml translation reaction mix containing Brome Mosaic Virus mRNA as template (following the manufacturer's protocol). Active ricin variants inhibit the *in vitro* translation reaction by inactivating ribosomes. Therefore, in the presence of an active ricin variant, no viral proteins are synthesized.

In Vitro Yeast Protein Synthesis Assay

The activity of protease-treated proricin-like toxins may also be assessed by a yeast protein synthesis assay. For example, Murakami, S et al., Mol., Cel. Biol. 2:588-592, 1982, teaches a yeast protein synthesis assay to determine ricin-like toxicity which is as sensitive as mammalian cell assays.

Six five mL cultures of *Saccharomyces cerevisiase* (Y235 cells and 2 cell wall mutants) in YPD medium (10 g/L yeast extract, 20 g/L peptone) are started by inoculating 800 uL of medium with 1 colony of *Saccharomyces cerevisiase*, vortexing, then adding 100 uL of this suspension to 5 mL of medium. Cultures are grown overnight at 30°C with gentle agitation. Cells are expanded by inoculating 100 uL of YPD medium with one or more of the 5 mL overnight cultures and are grown at 30°C with gentle agitation until a concentration of 1 x 10^5 cells/mL. Cells are washed with sterile double-distilled water, centrifuged at 1,200 g for 3 minutes and concentrated 3-fold in ZSM buffer(1 M sorbitol, 10 mM Tris-Cl, pH 7.5, 50 mM dithiotheitol (DDT)). Samples are incubated with gentle shaking for 10 minutes at 30°C, centrifuged at 1,200 g for 3 minutes and resuspended in ZSM buffer such that the cell concentration was 1 x 10^8 cells/ml. Cell walls are disrupted by adding 1 mL of beta-glucuronidase (Sigma, St. Louis, MO) to the samples and incubating for 1 hour at room temperature with gentle agitation. Cells are washed 3 times with ZSM and protoplast cells resuspended in regeneration medium (0.17% yeast nitrogen base without amino acids (Difco, Detroit, Michigan), 2 Dropout + all (essential amino acids), 10 mM Tris-Cl, pH 7.5, 2% glucose, 1M sorbitol) to a final concentration of 1 x 10^8 cells/mL. An activated proricin variant which has been dialysed in sterile 1X baculo buffer (0.137 M) NaCl, 2.7 mM KCl, 2.6 mM KH_2PO_4 pH 7.4) is added to one half of the protoplast, while sterile 1 X baculo buffer alone is added to the other half of the protoplasts as control. Both sets of samples are incubated at room temperature with gentle agitation. At time periods of 0, 1, 2, and 3 hours, an aliquot of each culture is removed. The cells are diluted serially from 10^{-4} to 10^{-8} in ZSM and plated on soft agar (1:1 ZSM:YPD, 15% agar). Simultaneously, dilutions are made from 10^{-2} to 10^{-4} in sterile double-distilled water and 50 uL aliquots are plated onto YPD medium with 20% agar. Plates are incubated for 2 days at 30°C after which times colonies were counted. A plot of cell count vs. time is used to compare the ricin test culture vs. the control culture with no ricin.

The activated proricin-like toxin variant inhibits *in vitro* protein synthesis through ribosomal inactivation. The rate of cell growth of the treatment group is expected to be substantially lower than that of the control group.

N-Glycosidase Activity of Proricin Variants on rRNA Oligonucleotides

Ricin-like toxins inhibit ribosomal function by hydrolysing the N-glycosidic bond between the nucleotide base and the ribose at position A4319 in eukaryotic 28S ribosomal

RNA (rRNA). The ability of the activated ricin-like toxins to inhibit ribosomal RNA (rRNA) function may be examined in an *in vitro* ribonucleotide catalysis assay using a synthetic oligoribonucleotide possessing the secondary structure of the natural RNA hydrolytic cleavage domain.

A synthetic 32-nucleotide RNA oligomer (University of Calgary, DNA Core Services) that mimics the 28S rRNA toxin active site is used to test the N-glycosidase activity of proricin variants. The sequence of oligonucleotide and the general methodology are substantially as described in Gluck, A. and Wool I.G., *J. Mol. Biol.* 256:838-848, 1996.

A labelling reaction is set up to include: 50 pmol of oligonucleotide, 20 units of T4 polynucleotide kinase (PNK; Gibco-BRL, Gaithersburg, MA), 25 pmol of γ-^{32}P (Amersham, Arlington, IL), 1X T4 PNK buffer in a final volume of 50 uL. The samples are incubated for 30 minutes at 37°C and them for 20 minutes at 65°C. The labelled oligonucleotide is precipitated with 95% ethanol an dried using a thermal cycler. A second ethanol precipitation step can be repeated to remove further trace contaminants. The RNA was resuspended to a final concentration of 1 ng/uL in 10 mM Tris-Cl (pH 7.6) and 50 mM NaCl (5 ng of oligonucleotide is used per sample).

Activated proricin variant is reduced in 1 X baculo buffer with 1% beta-mercaptoethanol for 30 minutes at room temperature prior to use. The oligonucleotides are heated at 90°C for 1 minute in 10 mM Tris-Cl (pH 7.6), 50 mM NaCl and allowed to renature at 0°C. $CaCl_2$, EGTA and water are added to the renatured RNA to give the following concentrations: 3 mM Tris-HCl (pH 7.6), 15 mM NaCl, 5 mM $CaCl_2$, and 5 mM EGTA. An activated proricin variant or ricin A-chain (Sigma, St. Louis, MO) is added to each tube. The concentration of the ricin ranged from 1-10 uM and the proricin variant 10-fold greater. The tubes are incubated at 35°C for 20 minutes and the reaction is stopped by the addition of sodium dodecylsulfate (SDS) at a final concentration of 0.5% (w/v). The oligonucleotide and 15 ug of added carrier tRNA (yeast tRNA; Gibco-BRL Gaithersburg, MA) are precipitated with 300 mM NaCl and 2.5 volumes of 95% ethanol. The pellets are washed once with 70% ethanol and dried on a CENTRIVAP (Labconco, Kansas City, MO). The RNA is dissolved in 5 uL of water, 25 uL of a solution of aniline and acetic acid (1 and 2.8 mM respectively) is added and the sample is incubated for 10 minutes at 40°C. The aniline-treated RNA is precipitate with ethanol and 300 mM NaCl, washed once in 70% ethanol and dried on the CENTRIVAP. The pellets are dissovled in 10 uL of DEPC-treated double-distilled water and 10 uL of 2X loading dy (178 mM Tris-HCl (pH 8.3), 178 mM boric acid, 5 mM EDTA, 0.05% (w/v) bromophenol blue and 14 M urea), and are electrophoresed for 3 hours at 50 watts in 10% (w/v) polyacrylamide gel containing 7 M

urea in 1 X TBE buffer (89 mM Tris-HCl (pH 8.3), 89 mM boric acid, 2.5 mM boric acid, 2.5 mM EDTA). Gels are exposed to KODAK full speed blue X-ray film and left at -70°C. After 2 days, film was developed in a KODAK automatic film processor.

When proricin variant activated with a disease-specific protease is added to the oligoribonucleotide, hydrolysis of the N- glycosidic bond at position 20 (depurination of adenosine) would occur and appearance of two bands on the autograph is expected. Proricin variant without pretreatment with the disease-specific protease would not cleave the RNA oligonucleotide and would result in a single band on the autoradiograph.

In Vitro Cytotoxicity Assay:

Human ovarian cancer cells (*e.g.* MA148) are seeded in 96-well flat-bottom plates and are exposed to ricin-like toxin variants or control medium at 37°C for 16 h. The viability of the cancer cells is determined by measuring [^{35}S]methionine incorporation and is significantly lower in wells treated with the toxin variants than those with control medium.

In Vivo Tumour Growth Inhibition Assay:

Human breast cancer (*e.g.* MCF-7) cells are maintained in suitable medium containing 10% fetal calf serum. The cells are grown, harvested and subsequently injected subcutaneously into ovariectomized athymic nude mice. Tumour size is determined at intervals by measuring two right-angle measurements using calipers.

In Vivo Tumour Metastasis Assay:

The metastasis study is performed substantially as described in Honn, K.V. et al. (*Biochem. Pharmacol.* 34:235-241 (1985)). Viable B16a melanoma tumour cells are prepared and injected subcutaneously into the left axillary region of syngeneic mice. The extent of tumour metastasis is measured after 4 weeks. The lungs are removed from the animals and are fixed in Bouin's solution and macroscopic pulmonary metastases are counted using a dissecting microscope. In general without therapeutic intervention, injection of 10^5 viable tumour cells forms approximately 40-50 pulmonary metastases.

Having illustrated and described the principles of the invention in a preferred embodiment, it should be appreciated to those skilled in the art that the invention can be modified in arrangement and detail without departure from such principles. We claim all modifications coming within the scope of the following claims.

All publications, patents and patent applications referred to herein are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

TABLE 1

Table 1 - Sequence and Location of Oligonucleotide Primers

Name of Primer	Primer Sequence†	Corresponds to proricin nucleotide numbers (see Figures 8-10)
Ricin-109	5'-GGAGATGAAACCGGGAGGAAATACTATTGTAAT-3'	27 to 59
Ricin-99Eco	5' - GCGGAATTCCGGGAGGAAATACTATTGTAAT - 3'	37 to 59
Ricin 267	5' - ACGGTTTATTTTAGTTGA - 3'	300 to 317
Ricin486	5' - ACTTGCTGGTAATCTGAG - 3'	519 to 536
Ricin 725	5' - AGAATAGTTGGGGGAGAC - 3'	758 to 775
Ricin937	5' - AATGCTGATGTTTGTATG - 3'	970 to 987
Ricin1151	5' - CGGGAGTCTATGTGATGA - 3'	1184 to 1201
Ricin1399	5' - GCAAATAGTGGACAAGTA - 3'	1432 to 1449
Ricin1627	5' - GGATTGGTGTTAGATGTG - 3'	1660 to 1677
Ricin1729C	5' - ATAACTTGCTGTCCTTTCA - 3'	1864 to 1846
Ricin1729C Xba	5' - CGCTCTAGATAACTTGCTGTCCTTTCA - 3'	1864 to 1846

† underlined sequences inserted for subcloning purposes and not included in final preproricin sequences

WE CLAIM:

1. A purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin- like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for a retroviral protease.

2. The nucleic acid of claim 1 wherein the A chain is ricin A chain.

3. The nucleic acid of claim 1 wherein the B chain is ricin B chain.

4. The nucleic acid of claim 1 wherein the cleavage recognition site is the cleavage recognition site for an HIV protease.

5. The nucleic acid of claim 1 wherein the linker amino acid sequence comprises VSQNYPIVQNFN; SKARVLAEAMSN; or SIRKILFLDGIN.

6. The nucleic acid of claim 1 having the nucleotide sequence shown in Figure 8, Figure 9 or Figure 10.

7. The nucleic acid of claim 1 wherein the cleavage recognition site is the cleavage recognition site for a human T-cell leukemia virus (HTLV) protease.

8. The nucleic acid of claim 7 wherein the linker amino acid sequence comprises SAPQVLPVMHPN; SKTKVLVVQPKN; SKTKVLVVQPRN or STTQCFPILHPN.

9. A plasmid incorporating the nucleic acid of claim 1.

10. A plasmid as claimed in claim 7 having the restriction map as shown in Figure 1A, 2A, 3A, 16A, 17A or 18A.

11. A baculovirus transfer vector incorporating the nucleic acid of claim 1.

12. A baculovirus transfer vector as claimed in claim 11 having the restriction map as shown in Figure 5, 6, 7, 16C, 17C, or 18C.

13. A baculovirus transfer vector as claimed in claim 11 having the DNA sequence as shown in Figure 11.

14. A recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for a retroviral protease.

15. The recombinant protein of claim 14 wherein the A chain is ricin A chain.

16. The recombinant protein of claim 14 wherein the B chain is ricin B chain.

17. The recombinant protein of claim 14 wherein the cleavage recognition site is the cleavage recognition site for an HIV protease.

18. The recombinant protein of claim 14 wherein the linker amino acid sequence comprises VSQNYPIVQNFN; SKARVLAEAMSN; or SIRKILFLDGIN.

19. A recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin- like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for a HTLV protease

20. The recombinant protein of claim 19 wherein the linker amino acid sequence comprises SAPQVLPVMHPN; SKTKVLVVQPKN; SKTKVLVVQPRN or STTQCFPILHPN.

21. A method of inhibiting or destroying mammalian cells infected with a retrovirus having a protease, comprising the steps of preparing a recombinant protein of claim 14 wherein the linker sequence contains a cleavage recognition site for the retrovirus protease and administering the fusion protein to the cells.

22. A method as claimed in claim 21, wherein the retrovirus is HIV.

23. A method as claimed in claim 21 wherein the mammalian cells are human cells.

24. A method of treating a mammal infected with HIV, comprising the steps of preparing a recombinant protein of claim 14 and administering the protein to the

mammal.

25. A process for preparing a pharmaceutical for treating a mammal infected with a retrovirus having a protease comprising the steps of preparing a purified and isolated nucleic acid having a nucleotide sequence encoding an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains a cleavage recognition site for the protease; introducing the nucleic acid into a host cell; expressing the nucleic acid in the host cell to obtain a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a linker amino acid sequence, linking the A and B chains wherein the linker sequence contains the cleavage recognition site for the protease, and suspending the protein in a pharmaceutically acceptable carrier, diluent or excipient.

26. A process for preparing a pharmaceutical for treating a mammal infected with a retrovirus having a protease comprising the steps of identifying a cleavage recognition site for the protease; preparing a recombinant protein comprising an A chain of a ricin-like toxin, a B chain of a ricin-like toxin and a heterologous linker amino acid sequence, linking the A and B chains, wherein the linker sequence contains the cleavage recognition site for the protease and suspending the protein in a pharmaceutically acceptable carrier, diluent or excipient.

27. A pharmaceutical composition for treating a retroviral infection in a mammal comprising the recombinant protein of claim 14 and a pharmaceutically acceptable carrier, diluent or excipient.

28. A pharmaceutical composition for treating HIV infection in a mammal comprising the recombinant protein of claim 14 and a pharmaceutically acceptable carrier, diluent or excipient.

1/47

FIGURE 1A

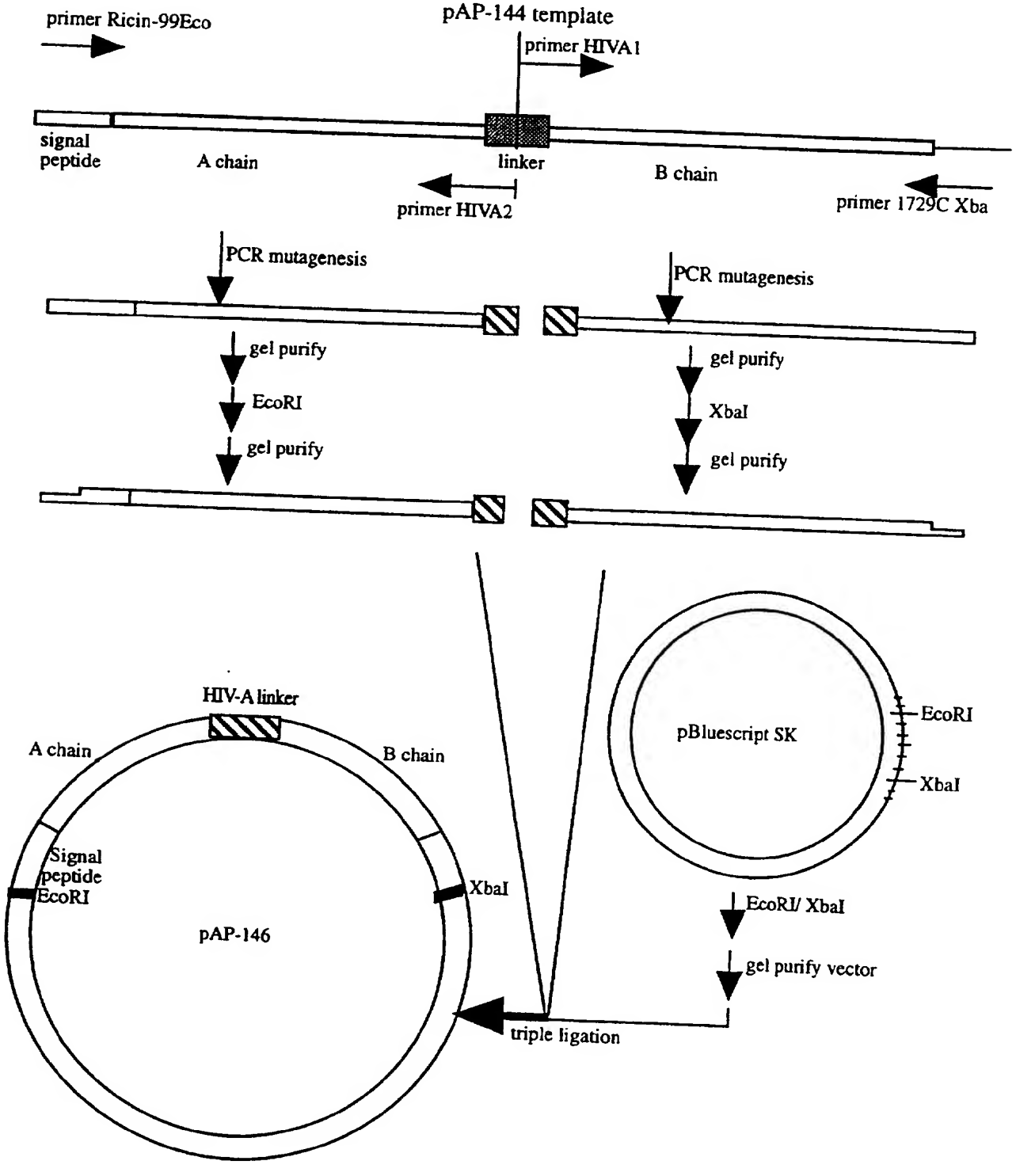


FIGURE 1B

WT preproricin linker
primer HIVA1
5'- TATCCAATAGTGCAAAATTTTAATGCTGAT -3'
TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT
AGAAACGAATATTCCGGTCACCACGGTTTAAAATTA
3'- GGTGGTAGCAGTGTCAAACAAAGCGTCTTG -5'
primer HIVA2
PCR mutagenesis
ligate with pBluescript SK
pAP146 linker
(HIV-A variant)
GTTTCGCAGAACTATCCAATAGTGCAAAATTTTAAT
CAAAGCGTCTTGATAGGTTATCACGTTTTAAAATTA

FIGURE 2A

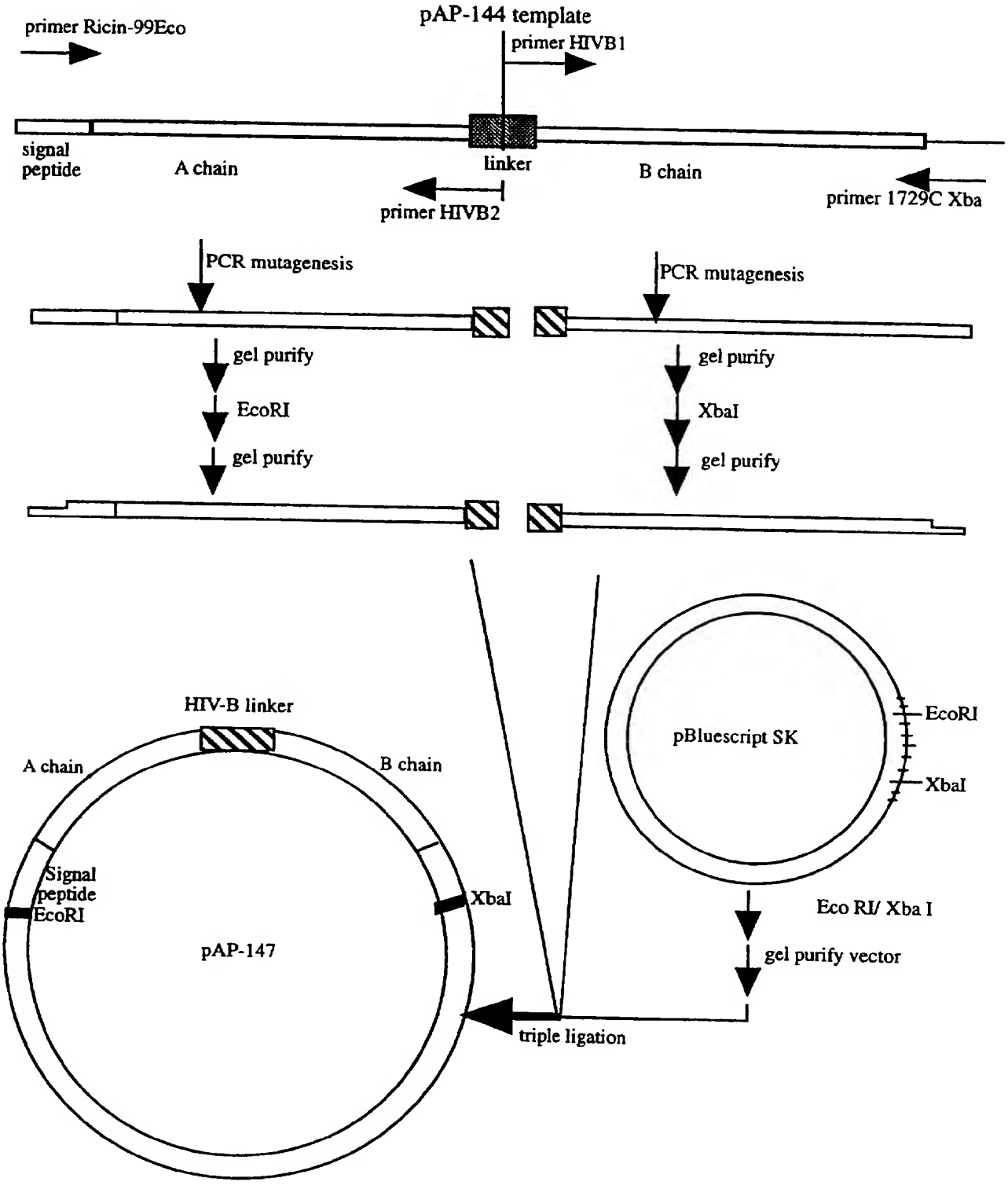
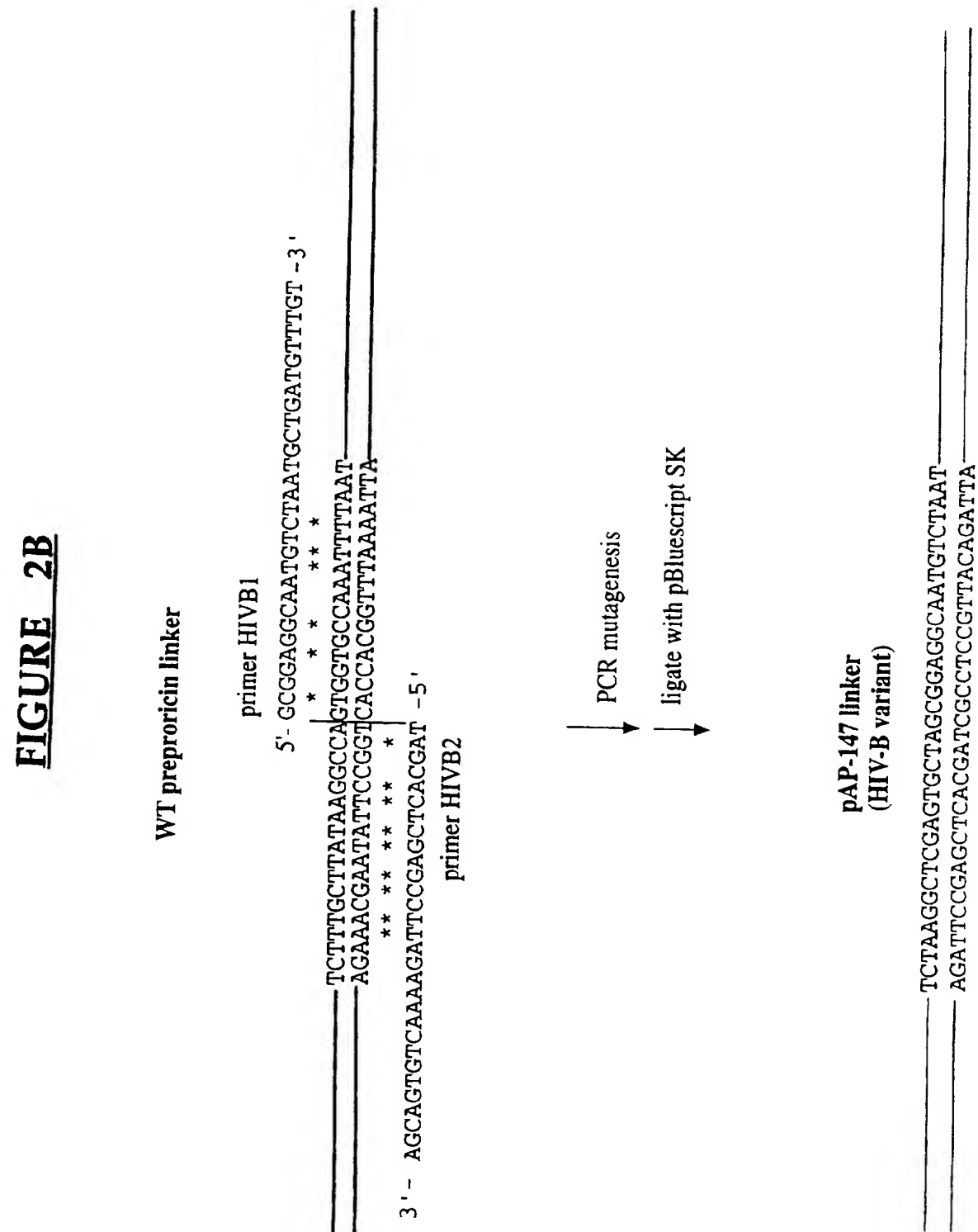

pAP-144 template
primer Ricin-99Eco
primer HIVB1
signal peptide
A chain
linker
B chain
primer HIVB2
primer 1729C Xba
PCR mutagenesis
PCR mutagenesis
gel purify
EcoRI
gel purify
gel purify
XbaI
gel purify
HIV-B linker
A chain
B chain
Signal peptide
EcoRI
XbaI
pAP-147
pBluescript SK
EcoRI
XbaI
Eco RI/ Xba I
gel purify vector
triple ligation

FIGURE 2B


WT preproricin linker
primer HIVB1
5'- GCGGAGGCAATGTCTAATGCTGATGTTTGT -3'
TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT
AGAAACGAATATTCCGGTCACCACGGTTTAAAATTA
3'- AGCAGTGTCAAAGATTCCGAGCTCACGAT -5'
primer HIVB2
PCR mutagenesis
ligate with pBluescript SK
pAP-147 linker
(HIV-B variant)
TCTAAGGCTCGAGTGCTAGCGGAGGCAATGTCTAAT
AGATTCCGAGCTCACGATCGCCTCCGTTACAGATTA

5/47

FIGURE 3A

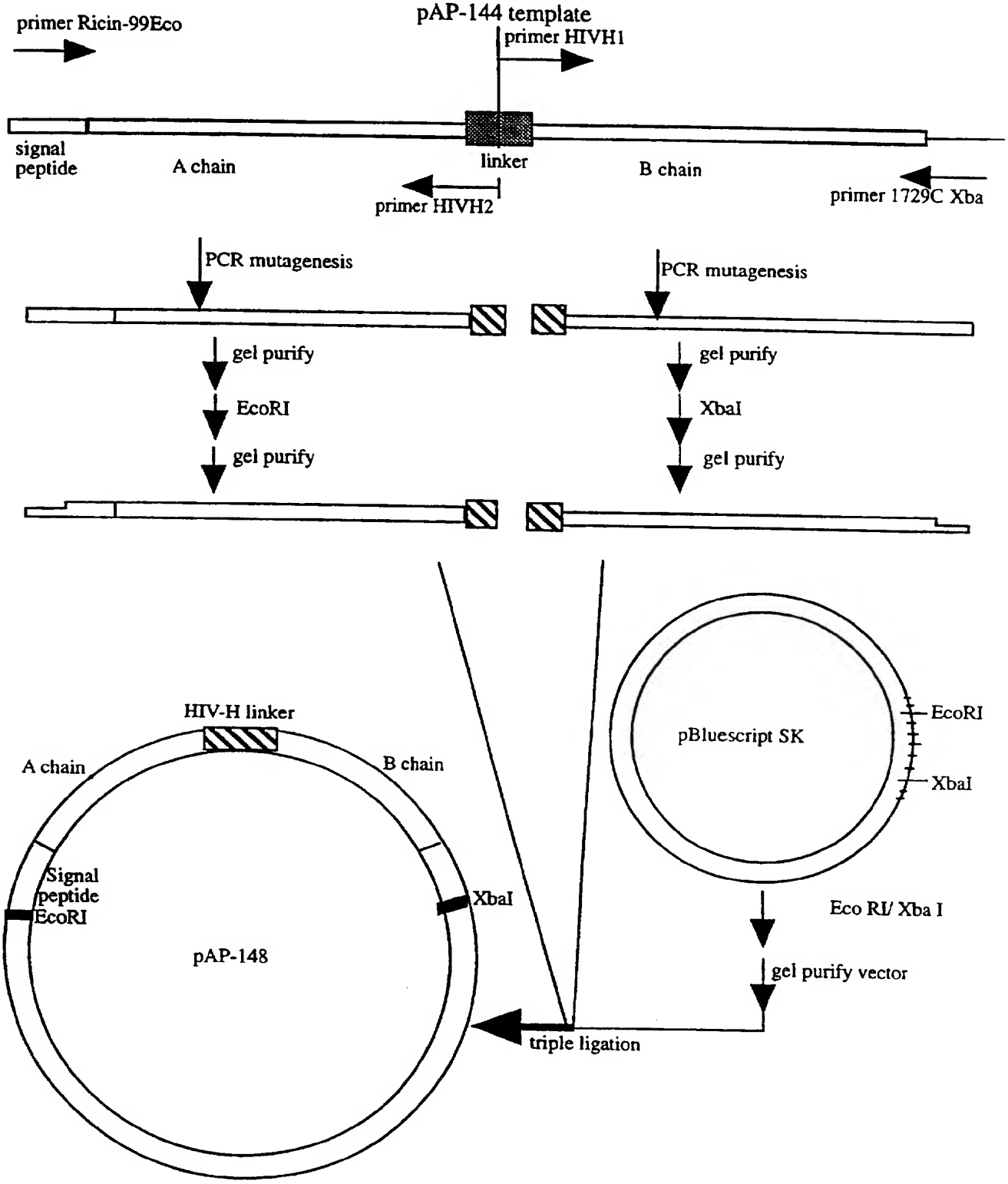
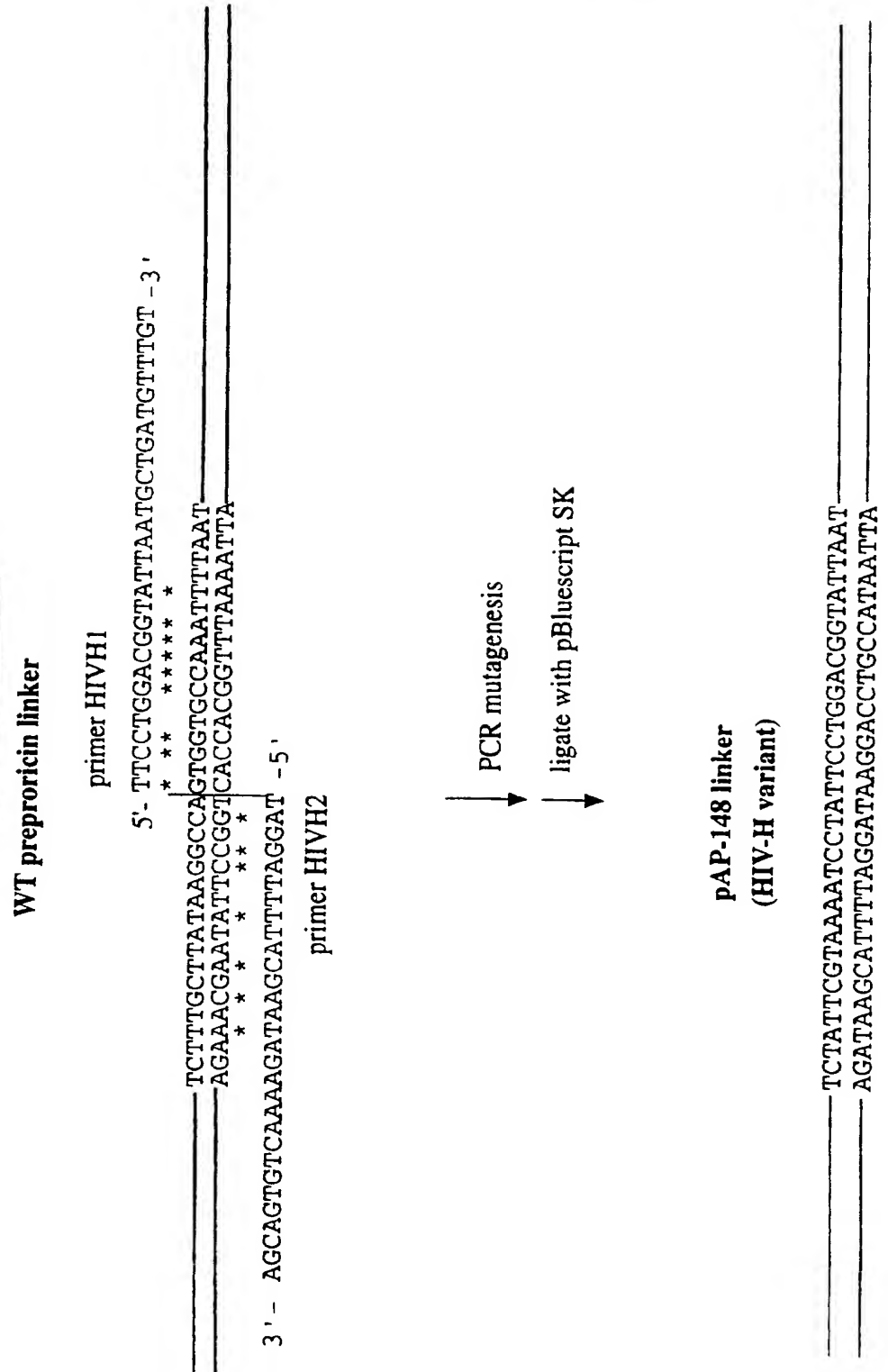


FIGURE 3B


WT preproricin linker
primer HIVH1
5'- TTCCTGGACGGTATTAATGCTGATGTTTGT -3'
* ** ***** *
TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTAAT
AGAAACGAATATTCCGGTCACCACGGTTTAAATTA
* * * * ** *
3' - AGCAGTGTCAAAAGATAAGCATTTTAGGAT -5'
primer HIVH2
PCR mutagenesis
ligate with pBluescript SK
pAP-148 linker
(HIV-H variant)
TCTATTCGTAAAATCCTATTCCTGGACGGTATTAAT
AGATAAGCATTTTAGGATAAGGACCTGCCATAATTA

7/47

FIGURE 4

Wild type Ricin linker:	A chain- S L L I R P V V P N F N -B chain
pAP-146 linker:	A chain- V S Q N Y P I V Q N F N -B chain
pAP-147 linker:	A chain- S K A R V L A E A M S N -B chain
pAP-148 linker:	A chain- S I R K I L F L D G I N -B chain

pAP-146= Ricin cDNA mutant with HIV-A protease linker sequence
pAP-147= Ricin cDNA mutant with HIV-B protease linker sequence
pAP-148= Ricin cDNA mutant with HIV-H protease linker sequence

FIGURE 5

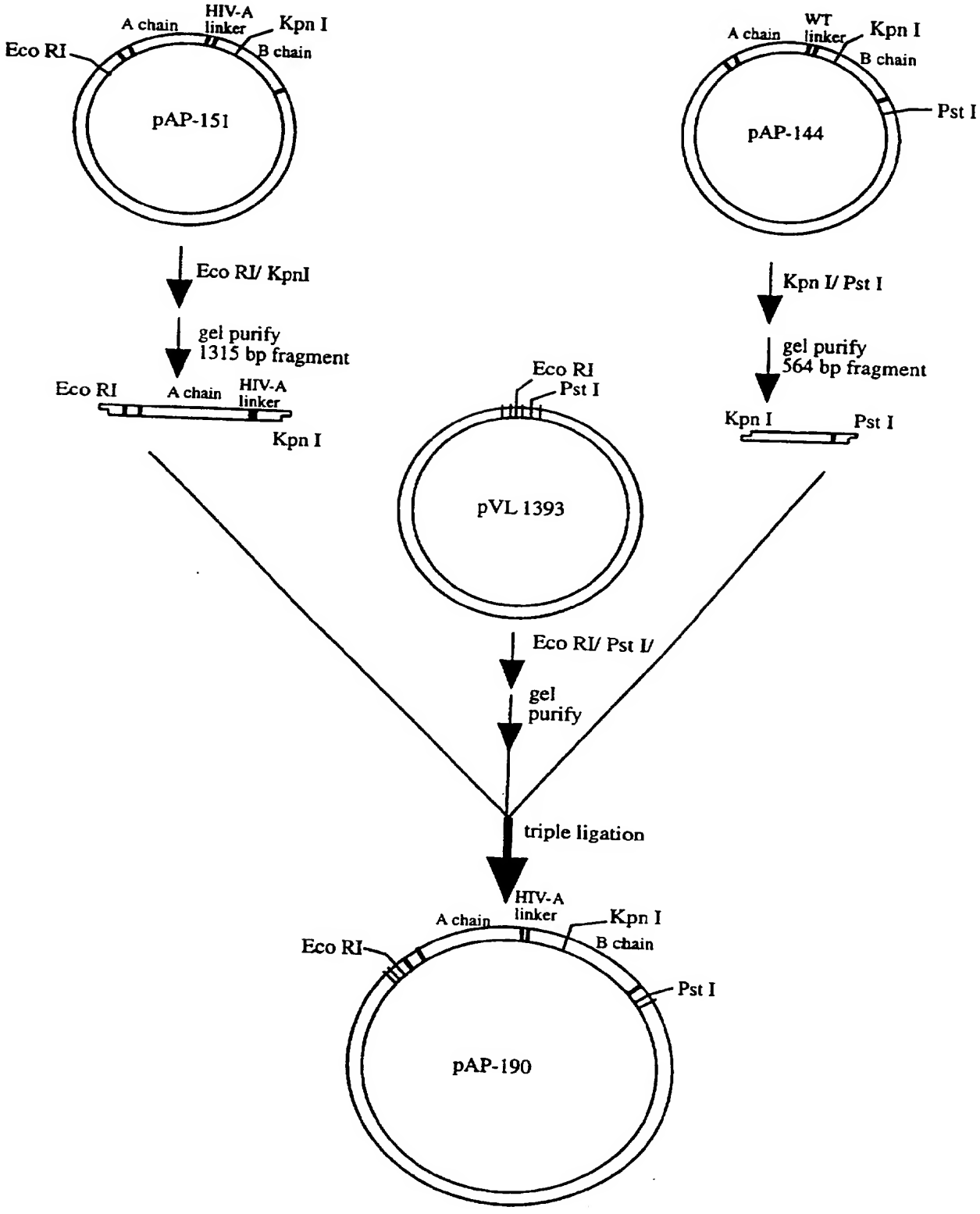

Eco RI
A chain
HIV-A linker
Kpn I
B chain
pAP-151
Eco RI/ KpnI
gel purify
1315 bp fragment
Eco RI
A chain
HIV-A linker
Kpn I
WT linker
Kpn I
A chain
B chain
Pst I
pAP-144
Kpn I/ Pst I
gel purify
564 bp fragment
Kpn I
Pst I
Eco RI
Pst I
pVL 1393
Eco RI/ Pst I/
gel purify
triple ligation
HIV-A linker
A chain
Kpn I
B chain
Eco RI
Pst I
pAP-190

FIGURE 6

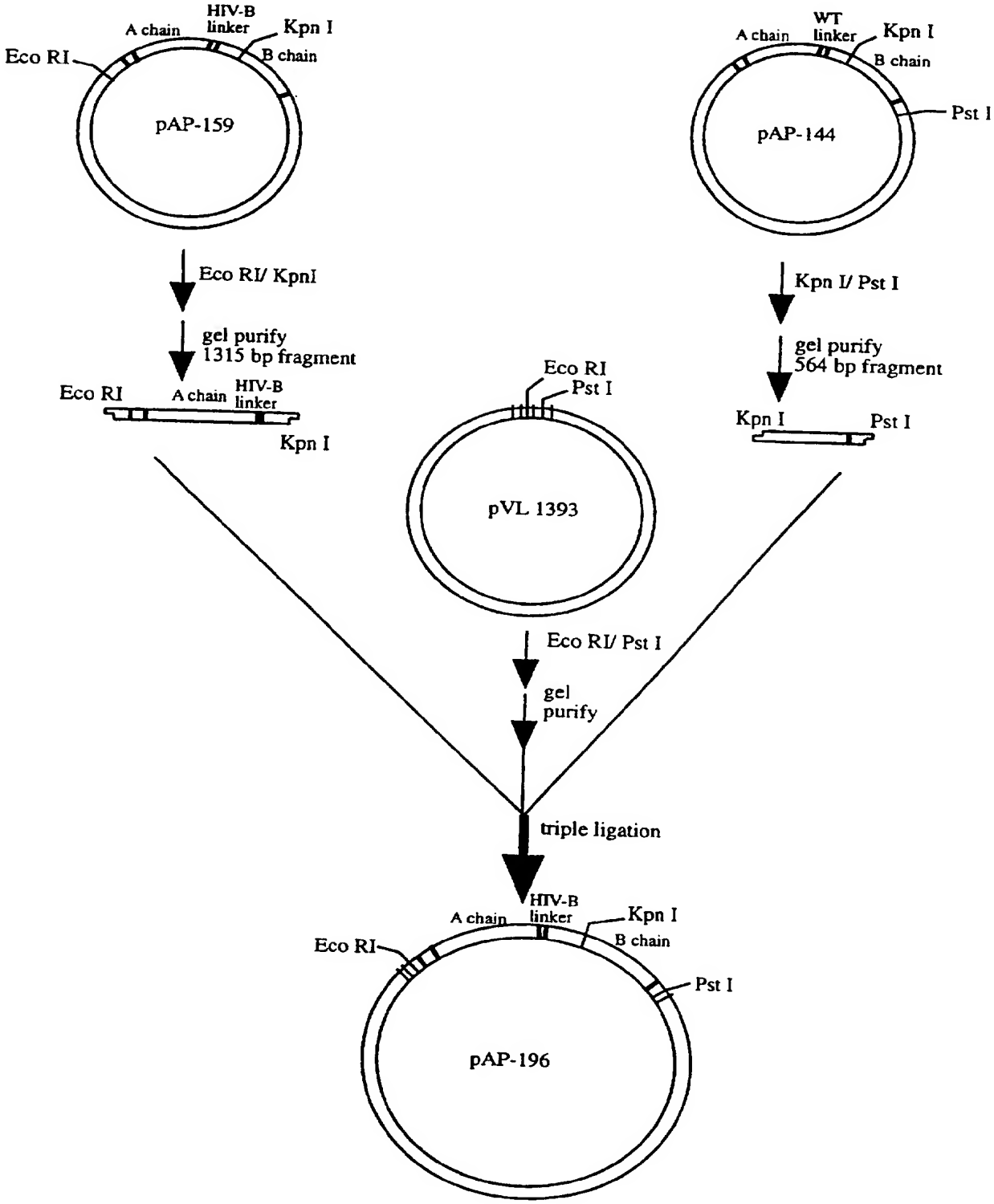

HIV-B linker
A chain
Kpn I
B chain
Eco RI
pAP-159
Eco RI/ KpnI
gel purify
1315 bp fragment
Eco RI
A chain
HIV-B linker
Kpn I
WT linker
A chain
Kpn I
B chain
Pst I
pAP-144
Kpn I/ Pst I
gel purify
564 bp fragment
Kpn I
Pst I
Eco RI
Pst I
pVL 1393
Eco RI/ Pst I
gel purify
triple ligation
HIV-B linker
A chain
Kpn I
B chain
Eco RI
Pst I
pAP-196

FIGURE 7

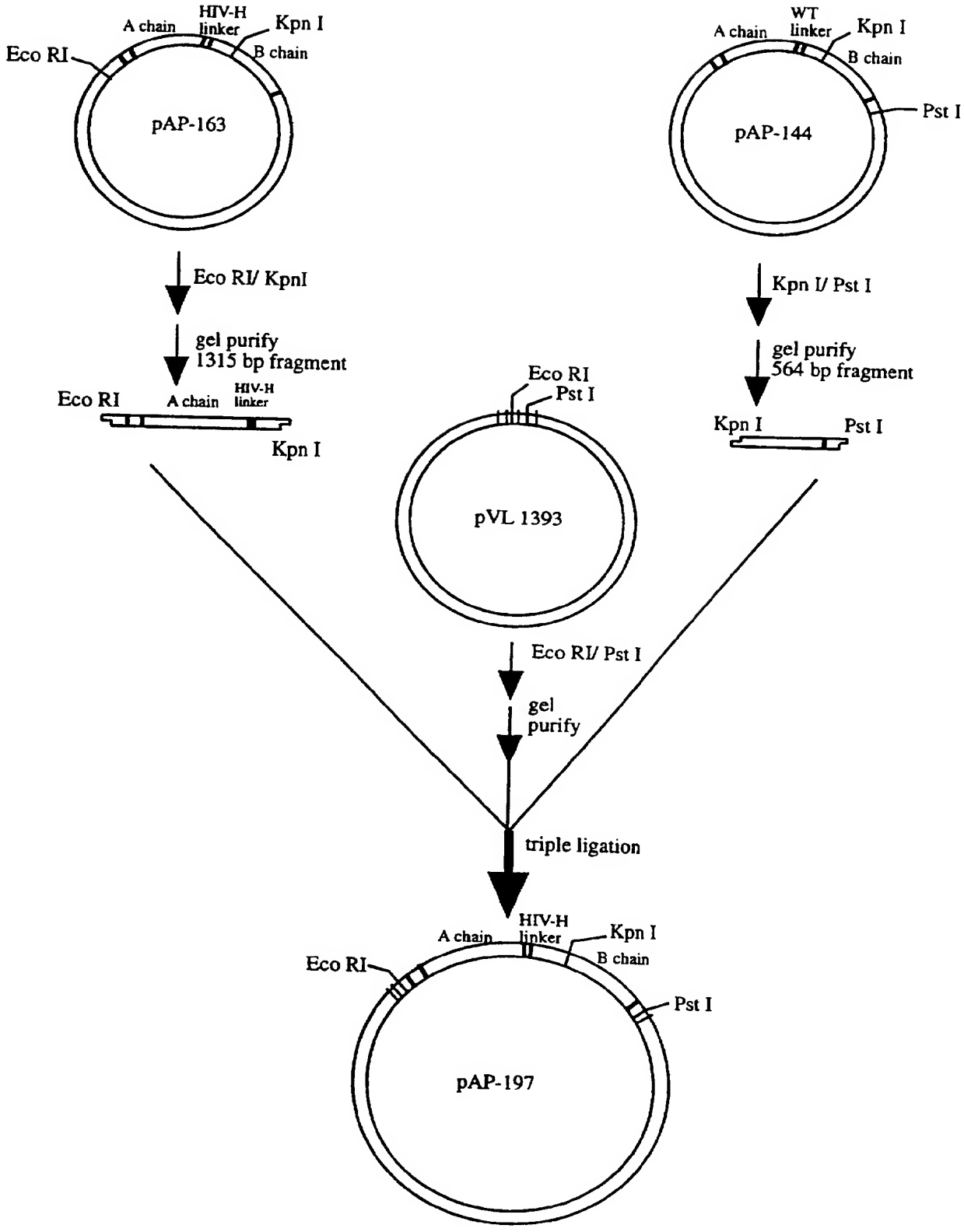

A chain
HIV-H linker
Kpn I
B chain
Eco RI
pAP-163
Eco RI/ KpnI
gel purify
1315 bp fragment
Eco RI
A chain
HIV-H linker
Kpn I
A chain
WT linker
Kpn I
B chain
Pst I
pAP-144
Kpn I/ Pst I
gel purify
564 bp fragment
Kpn I
Pst I
Eco RI
Pst I
pVL 1393
Eco RI/ Pst I
gel purify
triple ligation
A chain
HIV-H linker
Kpn I
B chain
Eco RI
Pst I
pAP-197

FIGURE 8

```
                10        20        30        40        50
                |         |         |         |         |
  1 GAATTCCCCTCGAGACGCGTCGACCCGGAGATGAAACCGGGAGGAAATAC
    CTTAAGGGGAGCTCTGCGCAGCTGGGCCTCTACTTTGGCCCTCCTTTATG

 51 TATTGTAATATGGATGTATGCAGTGGCAACATGGCTTTGTTTTGGATCCA
    ATAACATTATACCTACATACGTCACCGTTGTACCGAAACAAAACCTAGGT

101 CCTCAGGGTGGTCTTTCACATTAGAGGATAACAACATATTCCCCAAACAA
    GGAGTCCCACCAGAAAGTGTAATCTCCTATTGTTGTATAAGGGGTTTGTT

151 TACCCAATTATAAACTTTACCACAGCGGGTGCCACTGTGCAAAGCTACAC
    ATGGGTTAATATTTGAAATGGTGTCGCCCACGGTGACACGTTTCGATGTG

201 AAACTTTATCAGAGCTGTTCGCGGTCGTTTAACAACTGGAGCTGATGTGA
    TTTGAAATAGTCTCGACAAGCGCCAGCAAATTGTTGACCTCGACTACACT

251 GACATGATATACCAGTGTTGCCAAACAGAGTTGGTTTGCCTATAAACCAA
    CTGTACTATATGGTCACAACGGTTTGTCTCAACCAAACGGATATTTGGTT

301 CGGTTTATTTTAGTTGAACTCTCAAATCATGCAGAGCTTTCTGTTACATT
    GCCAAATAAAATCAACTTGAGAGTTTAGTACGTCTCGAAAGACAATGTAA

351 AGCGCTGGATGTCACCAATGCATATGTGGTCGGCTACCGTGCTGGAAATA
    TCGCGACCTACAGTGGTTACGTATACACCAGCCGATGGCACGACCTTTAT

401 GCGCATATTTCTTTCATCCTGACAATCAGGAAGATGCAGAAGCAATCACT
    CGCGTATAAAGAAAGTAGGACTGTTAGTCCTTCTACGTCTTCGTTAGTGA

451 CATCTTTTCACTGATGTTCAAAATCGATATACATTCGCCTTTGGTGGTAA
    GTAGAAAAGTGACTACAAGTTTTAGCTATATGTAAGCGGAAACCACCATT

501 TTATGATAGACTTGAACAACTTGCTGGTAATCTGAGAGAAAATATCGAGT
    AATACTATCTGAACTTGTTGAACGACCATTAGACTCTCTTTTATAGCTCA

551 TGGGAAATGGTCCACTAGAGGAGGCTATCTCAGCGCTTTATTATTACAGT
    ACCCTTTACCAGGTGATCTCCTCCGATAGAGTCGCGAAATAATAATGTCA

601 ACTGGTGGCACTCAGCTTCCAACTCTGGCTCGTTCCTTTATAATTTGCAT
    TGACCACCGTGAGTCGAAGGTTGAGACCGAGCAAGGAAATATTAAACGTA

651 CCAAATGATTTCAGAAGCAGCAAGATTCCAATATATTGAGGGAGAAATGC
    GGTTTACTAAAGTCTTCGTCGTTCTAAGGTTATATAACTCCCTCTTTACG

701 GCACGAGAATTAGGTACAACCGGAGATCTGCACCAGATCCTAGCGTAATT
    CGTGCTCTTAATCCATGTTGGCCTCTAGACGTGGTCTAGGATCGCATTAA

751 ACACTTGAGAATAGTTGGGGGGAGACTTTCCACTGCAATTCAAGAGTCTAA
    TGTGAACTCTTATCAACCCCCTCTGAAAGGTGACGTTAAGTTCTCAGATT

801 CCAAGGAGCCTTTGCTAGTCCAATTCAACTGCAAAGACGTAATGGTTCCA
    GGTTCCTCGGAAACGATCAGGTTAAGTTGACGTTTCTGCATTACCAAGGT

851 AATTCAGTGTGTACGATGTGAGTATATTAATCCCTATCATAGCTCTCATG
    TTAAGTCACACATGCTACACTCATATAATTAGGGATAGTATCGAGAGTAC

901 GTGTATAGATGCGCACCTCCACCATCGTCACAGTTTGTTTCGCAGAACTA
    CACATATCTACGCGTGGAGGTGGTAGCAGTGTCAAACAAAGCGTCTTGAT
```

FIGURE 8 (Cont'd)

```
951  TCCAATAGTGCAAAATTTTAATGCTGATGTTTGTATGGATCCTGAGCCCA
     AGGTTATCACGTTTTAAAATTACGACTACAAACATACCTAGGACTCGGGT

1001 TAGTGCGTATCGTAGGTCGAAATGGTCTATGTGTTGATGTTAGGGATGGA
     ATCACGCATAGCATCCAGCTTTACCAGATACACAACTACAATCCCTACCT

1051 AGATTCCACAACGGAAACGCAATACAGTTGTGGCCATGCAAGTCTAATAC
     TCTAAGGTGTTGCCTTTGCGTTATGTCAACACCGGTACGTTCAGATTATG

1101 AGATGCAAATCAGCTCTGGACTTTGAAAAGAGACAATACTATTCGATCTA
     TCTACGTTTAGTCGAGACCTGAAACTTTTCTCTGTTATGATAAGCTAGAT

1151 ATGGAAAGTGTTTAACTACTTACGGGTACAGTCCGGGAGTCTATGTGATG
     TACCTTTCACAAATTGATGAATGCCCATGTCAGGCCCTCAGATACACTAC

1201 ATCTATGATTGCAATACTGCTGCAACTGATGCCACCCGCTGGCAAATATG
     TAGATACTAACGTTATGACGACGTTGACTACGGTGGGCGACCGTTTATAC

1251 GGATAATGGAACCATCATAAATCCCAGATCTAGTCTAGTTTTAGCAGCGA
     CCTATTACCTTGGTAGTATTTAGGGTCTAGATCAGATCAAAATCGTCGCT

1301 CATCAGGGAACAGTGGTACCACACTTACAGTGCAAACCAACATTTATGCC
     GTAGTCCCTTGTCACCATGGTGTGAATGTCACGTTTGGTTGTAAATACGG

1351 GTTAGTCAAGGTTGGCTTCCTACTAATAATACACAACCTTTTGTTACAAC
     CAATCAGTTCCAACCGAAGGATGATTATTATGTGTTGGAAAACAATGTTG

1401 CATTGTTGGGCTATATGGTCTGTGCTTGCAAGCAAATAGTGGACAAGTAT
     GTAACAACCCGATATACCAGACACGAACGTTCGTTTATCACCTGTTCATA

1451 GGATAGAGGACTGTAGCAGTGAAAAGGCTGAACAACAGTGGGCTCTTTAT
     CCTATCTCCTGACATCGTCACTTTTCCGACTTGTTGTCACCCGAGAAATA

1501 GCAGATGGTTCAATACGTCCTCAGCAAAACCGAGATAATTGCCTTACAAG
     CGTCTACCAAGTTATGCAGGAGTCGTTTTGGCTCTATTAACGGAATGTTC

1551 TGATTCTAATATACGGGAAACAGTTGTTAAGATCCTCTCTTGTGGCCCTG
     ACTAAGATTATATGCCCTTTGTCAACAATTCTAGGAGAGAACACCGGGAC

1601 CATCCTCTGGCCAACGATGGATGTTCAAGAATGATGGAACCATTTTAAAT
     GTAGGAGACCGGTTGCTACCTACAAGTTCTTACTACCTTGGTAAAATTTA

1651 TTGTATAGTGGATTGGTGTTAGATGTGAGGCGATCGGATCCGAGCCTTAA
     AACATATCACCTAACCACAATCTACACTCCGCTAGCCTAGGCTCGGAATT

1701 ACAAATCATTCTTTACCCTCTCCATGGTGACCCAAACCAAATATGGTTAC
     TGTTTAGTAAGAAATGGGAGAGGTACCACTGGGTTTGGTTTATACCAATG

1751 CATTATTTTGATAGACAGATTACTCTCTTGCAGTGTGTGTGTCCTGCCAT
     GTAATAAAACTATCTGTCTAATGAGAGAACGTCACACACACAGGACGGTA

1801 GAAAATAGATGGCTTAAATAAAAAGGACATTGTAAATTTTGTAACTGAAA
     CTTTTATCTACCGAATTTATTTTTCCTGTAACATTTAAAACATTGACTTT

1851 GGACAGCAAGTTATATCGAATTCCTGCAG
     CCTGTCGTTCAATATAGCTTAAGGACGTC
```

FIGURE 9

```
                10        20        30        40        50
                |         |         |         |         |
  1 GAATTCCCCTCGAGACGCGTCGACCCGGAGATGAAACCGGGAGGAAATAC
    CTTAAGGGGAGCTCTGCGCAGCTGGGCCTCTACTTTGGCCCTCCTTTATG

 51 TATTGTAATATGGATGTATGCAGTGGCAACATGGCTTTGTTTTGGATCCA
    ATAACATTATACCTACATACGTCACCGTTGTACCGAAACAAAACCTAGGT

101 CCTCAGGGTGGTCTTTCACATTAGAGGATAACAACATATTCCCCAAACAA
    GGAGTCCCACCAGAAAGTGTAATCTCCTATTGTTGTATAAGGGGTTTGTT

151 TACCCAATTATAAACTTTACCACAGCGGGTGCCACTGTGCAAAGCTACAC
    ATGGGTTAATATTTGAAATGGTGTCGCCCACGGTGACACGTTTCGATGTG

201 AAACTTTATCAGAGCTGTTCGCGGTCGTTTAACAACTGGAGCTGATGTGA
    TTTGAAATAGTCTCGACAAGCGCCAGCAAATTGTTGACCTCGACTACACT

251 GACATGATATACCAGTGTTGCCAAACAGAGTTGGTTTGCCTATAAACCAA
    CTGTACTATATGGTCACAACGGTTTGTCTCAACCAAACGGATATTTGGTT

301 CGGTTTATTTTAGTTGAACTCTCAAATCATGCAGAGCTTTCTGTTACATT
    GCCAAATAAAATCAACTTGAGAGTTTAGTACGTCTCGAAAGACAATGTAA

351 AGCGCTGGATGTCACCAATGCATATGTGGTCGGCTACCGTGCTGGAAATA
    TCGCGACCTACAGTGGTTACGTATACACCAGCCGATGGCACGACCTTTAT

401 GCGCATATTTCTTTCATCCTGACAATCAGGAAGATGCAGAAGCAATCACT
    CGCGTATAAAGAAAGTAGGACTGTTAGTCCTTCTACGTCTTCGTTAGTGA

451 CATCTTTTCACTGATGTTCAAAATCGATATACATTCGCCTTTGGTGGTAA
    GTAGAAAAGTGACTACAAGTTTTAGCTATATGTAAGCGGAAACCACCATT

501 TTATGATAGACTTGAACAACTTGCTGGTAATCTGAGAGAAAATATCGAGT
    AATACTATCTGAACTTGTTGAACGACCATTAGACTCTCTTTTATAGCTCA

551 TGGGAAATGGTCCACTAGAGGAGGCTATCTCAGCGCTTTATTATTACAGT
    ACCCTTTACCAGGTGATCTCCTCCGATAGAGTCGCGAAATAATAATGTCA

601 ACTGGTGGCACTCAGCTTCCAACTCTGGCTCGTTCCTTTATAATTTGCAT
    TGACCACCGTGAGTCGAAGGTTGAGACCGAGCAAGGAAATATTAAACGTA

651 CCAAATGATTTCAGAAGCAGCAAGATTCCAATATATTGAGGGAGAAATGC
    GGTTTACTAAAGTCTTCGTCGTTCTAAGGTTATATAACTCCCTCTTTACG

701 GCACGAGAATTAGGTACAACCGGAGATCTGCACCAGATCCTAGCGTAATT
    CGTGCTCTTAATCCATGTTGGCCTCTAGACGTGGTCTAGGATCGCATTAA

751 ACACTTGAGAATAGTTGGGGGAGACTTTCCACTGCAATTCAAGAGTCTAA
    TGTGAACTCTTATCAACCCCCTCTGAAAGGTGACGTTAAGTTCTCAGATT

801 CCAAGGAGCCTTTGCTAGTCCAATTCAACTGCAAAGACGTAATGGTTCCA
    GGTTCCTCGGAAACGATCAGGTTAAGTTGACGTTTCTGCATTACCAAGGT

851 AATTCAGTGTGTACGATGTGAGTATATTAATCCCTATCATAGCTCTCATG
    TTAAGTCACACATGCTACACTCATATAATTAGGGATAGTATCGAGAGTAC

901 GTGTATAGATGCGCACCTCCACCATCGTCACAGTTTTCTAAGGCTCGAGT
    CACATATCTACGCGTGGAGGTGGTAGCAGTGTCAAAAGATTCCGAGCTCA

951 GCTAGCGGAGGCAATGTCTAATGCTGATGTTTGTATGGATCCTGAGCCCA
```

FIGURE 9 (Cont'd)

```
      CGATCGCCTCCGTTACAGATTACGACTACAAACATACCTAGGACTCGGGT

1001  TAGTGCGTATCGTAGGTCGAAATGGTCTATGTGTTGATGTTAGGGATGGA
      ATCACGCATAGCATCCAGCTTTACCAGATACACAACTACAATCCCTACCT

1051  AGATTCCACAACGGAAACGCAATACAGTTGTGGCCATGCAAGTCTAATAC
      TCTAAGGTGTTGCCTTTGCGTTATGTCAACACCGGTACGTTCAGATTATG

1101  AGATGCAAATCAGCTCTGGACTTTGAAAAGAGACAATACTATTCGATCTA
      TCTACGTTTAGTCGAGACCTGAAACTTTTCTCTGTTATGATAAGCTAGAT

1151  ATGGAAAGTGTTTAACTACTTACGGGTACAGTCCGGGAGTCTATGTGATG
      TACCTTTCACAAATTGATGAATGCCCATGTCAGGCCCTCAGATACACTAC

1201  ATCTATGATTGCAATACTGCTGCAACTGATGCCACCCGCTGGCAAATATG
      TAGATACTAACGTTATGACGACGTTGACTACGGTGGGCGACCGTTTATAC

1251  GGATAATGGAACCATCATAAATCCCAGATCTAGTCTAGTTTTAGCAGCGA
      CCTATTACCTTGGTAGTATTTAGGGTCTAGATCAGATCAAAATCGTCGCT

1301  CATCAGGGAACAGTGGTACCACACTTACAGTGCAAACCAACATTTATGCC
      GTAGTCCCTTGTCACCATGGTGTGAATGTCACGTTTGGTTGTAAATACGG

1351  GTTAGTCAAGGTTGGCTTCCTACTAATAATACACAACCTTTTGTTACAAC
      CAATCAGTTCCAACCGAAGGATGATTATTATGTGTTGGAAAACAATGTTG

1401  CATTGTTGGGCTATATGGTCTGTGCTTGCAAGCAAATAGTGGACAAGTAT
      GTAACAACCCGATATACCAGACACGAACGTTCGTTTATCACCTGTTCATA

1451  GGATAGAGGACTGTAGCAGTGAAAAGGCTGAACAACAGTGGGCTCTTTAT
      CCTATCTCCTGACATCGTCACTTTTCCGACTTGTTGTCACCCGAGAAATA

1501  GCAGATGGTTCAATACGTCCTCAGCAAAACCGAGATAATTGCCTTACAAG
      CGTCTACCAAGTTATGCAGGAGTCGTTTTGGCTCTATTAACGGAATGTTC

1551  TGATTCTAATATACGGGAAACAGTTGTTAAGATCCTCTCTTGTGGCCCTG
      ACTAAGATTATATGCCCTTTGTCAACAATTCTAGGAGAGAACACCGGGAC

1601  CATCCTCTGGCCAACGATGGATGTTCAAGAATGATGGAACCATTTTAAAT
      GTAGGAGACCGGTTGCTACCTACAAGTTCTTACTACCTTGGTAAAATTTA

1651  TTGTATAGTGGATTGGTGTTAGATGTGAGGCGATCGGATCCGAGCCTTAA
      AACATATCACCTAACCACAATCTACACTCCGCTAGCCTAGGCTCGGAATT

1701  ACAAATCATTCTTTACCCTCTCCATGGTGACCCAAACCAAATATGGTTAC
      TGTTTAGTAAGAAATGGGAGAGGTACCACTGGGTTTGGTTTATACCAATG

1751  CATTATTTTGATAGACAGATTACTCTCTTGCAGTGTGTGTGTCCTGCCAT
      GTAATAAAACTATCTGTCTAATGAGAGAACGTCACACACACAGGACGGTA

1801  GAAAATAGATGGCTTAAATAAAAAGGACATTGTAAATTTTGTAACTGAAA
      CTTTTATCTACCGAATTTATTTTTCCTGTAACATTTAAAACATTGACTTT

1851  GGACAGCAAGTTATATCGAATTCCTGCAG
      CCTGTCGTTCAATATAGCTTAAGGACGTC
```

15/47

FIGURE 10

```
                10        20        30        40        50
                |         |         |         |         |
  1 GAATTCCCCTCGAGACGCGTCGACCCGGAGATGAAACCGGGAGGAAATAC
    CTTAAGGGGAGCTCTGCGCAGCTGGGCCTCTACTTTGGCCCTCCTTTATG

 51 TATTGTAATATGGATGTATGCAGTGGCAACATGGCTTTGTTTTGGATCCA
    ATAACATTATACCTACATACGTCACCGTTGTACCGAAACAAAACCTAGGT

101 CCTCAGGGTGGTCTTTCACATTAGAGGATAACAACATATTCCCCAAACAA
    GGAGTCCCACCAGAAAGTGTAATCTCCTATTGTTGTATAAGGGGTTTGTT

151 TACCCAATTATAAACTTTACCACAGCGGGTGCCACTGTGCAAAGCTACAC
    ATGGGTTAATATTTGAAATGGTGTCGCCCACGGTGACACGTTTCGATGTG

201 AAACTTTATCAGAGCTGTTCGCGGTCGTTTAACAACTGGAGCTGATGTGA
    TTTGAAATAGTCTCGACAAGCGCCAGCAAATTGTTGACCTCGACTACACT

251 GACATGATATACCAGTGTTGCCAAACAGAGTTGGTTTGCCTATAAACCAA
    CTGTACTATATGGTCACAACGGTTTGTCTCAACCAAACGGATATTTGGTT

301 CGGTTTATTTTAGTTGAACTCTCAAATCATGCAGAGCTTTCTGTTACATT
    GCCAAATAAAATCAACTTGAGAGTTTAGTACGTCTCGAAAGACAATGTAA

351 AGCGCTGGATGTCACCAATGCATATGTGGTCGGCTACCGTGCTGGAAATA
    TCGCGACCTACAGTGGTTACGTATACACCAGCCGATGGCACGACCTTTAT

401 GCGCATATTTCTTTCATCCTGACAATCAGGAAGATGCAGAAGCAATCACT
    CGCGTATAAAGAAAGTAGGACTGTTAGTCCTTCTACGTCTTCGTTAGTGA

451 CATCTTTTCACTGATGTTCAAAATCGATATACATTCGCCTTTGGTGGTAA
    GTAGAAAAGTGACTACAAGTTTTAGCTATATGTAAGCGGAAACCACCATT

501 TTATGATAGACTTGAACAACTTGCTGGTAATCTGAGAGAAAATATCGAGT
    AATACTATCTGAACTTGTTGAACGACCATTAGACTCTCTTTTATAGCTCA

551 TGGGAAATGGTCCACTAGAGGAGGCTATCTCAGCGCTTTATTATTACAGT
    ACCCTTTACCAGGTGATCTCCTCCGATAGAGTCGCGAAATAATAATGTCA

601 ACTGGTGGCACTCAGCTTCCAACTCTGGCTCGTTCCTTTATAATTTGCAT
    TGACCACCGTGAGTCGAAGGTTGAGACCGAGCAAGGAAATATTAAACGTA

651 CCAAATGATTTCAGAAGCAGCAAGATTCCAATATATTGAGGGAGAAATGC
    GGTTTACTAAAGTCTTCGTCGTTCTAAGGTTATATAACTCCCTCTTTACG

701 GCACGAGAATTAGGTACAACCGGAGATCTGCACCAGATCCTAGCGTAATT
    CGTGCTCTTAATCCATGTTGGCCTCTAGACGTGGTCTAGGATCGCATTAA

751 ACACTTGAGAATAGTTGGGGGAGACTTTCCACTGCAATTCAAGAGTCTAA
    TGTGAACTCTTATCAACCCCCTCTGAAAGGTGACGTTAAGTTCTCAGATT

801 CCAAGGAGCCTTTGCTAGTCCAATTCAACTGCAAAGACGTAATGGTTCCA
    GGTTCCTCGGAAACGATCAGGTTAAGTTGACGTTTCTGCATTACCAAGGT

851 AATTCAGTGTGTACGATGTGAGTATATTAATCCCTATCATAGCTCTCATG
    TTAAGTCACACATGCTACACTCATATAATTAGGGATAGTATCGAGAGTAC

901 GTGTATAGATGCGCACCTCCACCATCGTCACAGTTTTCTATTCGTAAAAT
    CACATATCTACGCGTGGAGGTGGTAGCAGTGTCAAAAGATAAGCATTTTA

951 CCTATTCCTGGACGGTATTAATGCTGATGTTTGTATGGATCCTGAGCCCA
```

FIGURE 10 (Cont'd)

```
      GGATAAGGACCTGCCATAATTACGACTACAAACATACCTAGGACTCGGGT

1001  TAGTGCGTATCGTAGGTCGAAATGGTCTATGTGTTGATGTTAGGGATGGA
      ATCACGCATAGCATCCAGCTTTACCAGATACACAACTACAATCCCTACCT

1051  AGATTCCACAACGGAAACGCAATACAGTTGTGGCCATGCAAGTCTAATAC
      TCTAAGGTGTTGCCTTTGCGTTATGTCAACACCGGTACGTTCAGATTATG

1101  AGATGCAAATCAGCTCTGGACTTTGAAAAGAGACAATACTATTCGATCTA
      TCTACGTTTAGTCGAGACCTGAAACTTTTCTCTGTTATGATAAGCTAGAT

1151  ATGGAAAGTGTTTAACTACTTACGGGTACAGTCCGGGAGTCTATGTGATG
      TACCTTTCACAAATTGATGAATGCCCATGTCAGGCCCTCAGATACACTAC

1201  ATCTATGATTGCAATACTGCTGCAACTGATGCCACCCGCTGGCAAATATG
      TAGATACTAACGTTATGACGACGTTGACTACGGTGGGCGACCGTTTATAC

1251  GGATAATGGAACCATCATAAATCCCAGATCTAGTCTAGTTTTAGCAGCGA
      CCTATTACCTTGGTAGTATTTAGGGTCTAGATCAGATCAAAATCGTCGCT

1301  CATCAGGGAACAGTGGTACCACACTTACAGTGCAAACCAACATTTATGCC
      GTAGTCCCTTGTCACCATGGTGTGAATGTCACGTTTGGTTGTAAATACGG

1351  GTTAGTCAAGGTTGGCTTCCTACTAATAATACACAACCTTTTGTTACAAC
      CAATCAGTTCCAACCGAAGGATGATTATTATGTGTTGGAAAACAATGTTG

1401  CATTGTTGGGCTATATGGTCTGTGCTTGCAAGCAAATAGTGGACAAGTAT
      GTAACAACCCGATATACCAGACACGAACGTTCGTTTATCACCTGTTCATA

1451  GGATAGAGGACTGTAGCAGTGAAAAGGCTGAACAACAGTGGGCTCTTTAT
      CCTATCTCCTGACATCGTCACTTTTCCGACTTGTTGTCACCCGAGAAATA

1501  GCAGATGGTTCAATACGTCCTCAGCAAAACCGAGATAATTGCCTTACAAG
      CGTCTACCAAGTTATGCAGGAGTCGTTTTGGCTCTATTAACGGAATGTTC

1551  TGATTCTAATATACGGGAAACAGTTGTTAAGATCCTCTCTTGTGGCCCTG
      ACTAAGATTATATGCCCTTTGTCAACAATTCTAGGAGAGAACACCGGGAC

1601  CATCCTCTGGCCAACGATGGATGTTCAAGAATGATGGAACCATTTTAAAT
      GTAGGAGACCGGTTGCTACCTACAAGTTCTTACTACCTTGGTAAAATTTA

1651  TTGTATAGTGGATTGGTGTTAGATGTGAGGCGATCGGATCCGAGCCTTAA
      AACATATCACCTAACCACAATCTACACTCCGCTAGCCTAGGCTCGGAATT

1701  ACAAATCATTCTTTACCCTCTCCATGGTGACCCAAACCAAATATGGTTAC
      TGTTTAGTAAGAAATGGGAGAGGTACCACTGGGTTTGGTTTATACCAATG

1751  CATTATTTTGATAGACAGATTACTCTCTTGCAGTGTGTGTGTCCTGCCAT
      GTAATAAAACTATCTGTCTAATGAGAGAACGTCACACACACAGGACGGTA

1801  GAAAATAGATGGCTTAAATAAAAAGGACATTGTAAATTTTGTAACTGAAA
      CTTTTATCTACCGAATTTATTTTTCCTGTAACATTTAAAACATTGACTTT

1851  GGACAGCAAGTTATATCGAATTCCTGCAG
      CCTGTCGTTCAATATAGCTTAAGGACGTC
```

FIGURE 11

```
ID   PVL1393    preliminary; circular DNA; SYN;
9632 BP.
XX
AC   IG1137;
XX
DT   01-FEB-1993 (Rel. 7, Created)
DT   01-JUL-1995 (Rel. 12, Last updated, Version
1)
XX
DE   E. coli plasmid vector pVL1393 - complete.
XX
KW   cloning vector.
XX
OS   Cloning vector
OC   Artificial sequences; Cloning vehicles.
XX
RN   [1]
RC   p2Bac from baculovirus
RC   p2Blue from p2Bac
RC   pBlueBac from AcNPV
RC   pBlueBac2 from AcNPV
RC   pBlueBacIII from AcNPV
RC   pBlueBacHisA from AcNPV
RC   pBlueBacHisB from AcNPV
RC   pBlueBacHisC from AcNPV
RC   pVL1392, pVL1393 from pAc360
RA   ;
RT   ;
RL   The Digest 5:2-2(1992).
XX
CC   NM (pVL1393)
CC   CM (yes)
CC   NA (ds-DNA)
CC   TP (circular)
CC   ST ()
CC   TY (plasmid)
CC   SP (British
Biotechnology)(Invitrogen)
CC   HO (E.coli NM522)(E.coli
INValphaF')(insect)
CC   CP ()
CC   FN (expression)(transfer)
CC   SE ()
CC   PA (pAC360)
CC   BR (pVL1392)
CC   OF ()
CC   OR ()
XX
FH   Key              Location/Qualifiers
FH
```

FIGURE 11 (Cont'd)

```
FT   misc_feature      0..0
FT                     /note="1. pAc360, ori/amp/AcMNPV
polyhedrin gene
FT                     -> pVL1393 9632bp"
FT   transposon        0..0
FT                     /note="TRN AcMNPV"
FT   misc_binding      868..868
FT                     /note="SIT SacII"
FT   misc_binding      1395..1395
FT                     /note="SIT ApaI"
FT   misc_binding      1901..1901
FT                     /note="SIT XhoI"
FT   promoter          0..0
FT                     /note="PRO AcMNPV polyhedrin gene"
FT   misc_binding      0..0
FT                     /note="MCS
FT                     BamHI-SmaI-XbaI-EcoRI-NotI-XmaIII-PstI-
BglII"
FT   rep_origin        0..0
FT                     /note="ORI E. coli pMB1 (ColE1 and
pBR322)"
FT   CDS               complement(0..0)
FT                     /note="ANT E. coli beta-lactamase gene
(bla)
FT                     ampicillin resistance gene (apr/amp)"
XX
SQ   Sequence 9632 BP; 2602 A; 2122 C; 2176 G; 2732 T; 0
other;
     aagctttact cgtaaagcga gttgaaggat catatttagt tgcgtttatg
     agataagatt gaaagcacgt gtaaaatgtt tcccgcgcgt tggcacaact
     atttacaatg cggccaagtt ataaaagatt ctaatctgat atgttttaaa
     acacctttgc ggcccgagtt gtttgcgtac gtgactagcg aagaagatgt
     gtggaccgca gaacagatag taaaacaaaa ccctagtatt ggagcaataa
     tcgatttaac caacacgtct aaatattatg atggtgtgca ttttttgcgg
     gcgggcctgt tatacaaaaa aattcaagta cctggccaga ctttgccgcc
     tgaaagcata gttcaagaat ttattgacac ggtaaaagaa tttacagaaa
     agtgtcccgg catgttggtg ggcgtgcact gcacacacgg tattaatcgc
     accggttaca tggtgtgcag atatttaatg cacaccctgg gtattgcgcc
     gcaggaagcc atagatagat tcgaaaaagc cagaggtcac aaaattgaaa
     gacaaaatta cgttcaagat ttattaattt aattaatatt atttgcattc
     tttaacaaat actttatcct attttcaaat tgttgcgctt cttccagcga
     accaaaacta tgcttcgctt gctccgttta gcttgtagcc gatcagtggc
     gttgttccaa tcgacggtag gattaggccg gatattctcc accacaatgt
     tggcaacgtt gatgttacgt ttatgctttt ggttttccac gtacgtcttt
     tggccggtaa tagccgtaaa cgtagtgccg tcgcgcgtca cgcacaacac
     cggatgtttg cgcttgtccg cggggtattg aaccgcgcga tccgacaaat
     ccaccacttt ggcaactaaa tcggtgacct gcgcgtcttt tttctgcatt
     atttcgtctt tcttttgcat ggtttcctgg aagccggtgt acatgcggtt
     tagatcagtc atgacgcgcg tgacctgcaa atctttggcc tcgatctgct
     tgtccttgat ggcaacgatg cgttcaataa actcttgttt tttaacaagt
     tcctcggttt tttgcgccac caccgcttgc agcgcgtttg tgtgctcggt
     gaatgtcgca atcagcttag tcaccaactg tttgctctcc tcctcccgtt
     gtttgatcgc gggatcgtac ttgccggtgc agagcacttg aggaattact
     tcttctaaaa gccattcttg taattctatg gcgtaaggca atttggactt
```

FIGURE 11 (Cont'd)

cataatcagc tgaatcacgc cggatttagt aatgagcact gtatgcggct
gcaaatacag cgggtcgccc cttttcacga cgctgttaga ggtagggccc
ccattttgga tggtctgctc aaataacgat ttgtatttat tgtctacatg
aacacgtata gctttatcac aaactgtata ttttaaactg ttagcgacgt
ccttggccac gaaccggacc tgttggtcgc gctctagcac gtaccgcagg
ttgaacgtat cttctccaaa tttaaattct ccaattttaa cgcgagccat
tttgatacac gtgtgtcgat tttgcaacaa ctattgtttt ttaacgcaaa
ctaaacttat tgtggtaagc aataattaaa tatgggggaa catgcgccgc
tacaacactc gtcgttatga acgcagacgg cgccggtctc ggcgcaagcg
gctaaaacgt gttgcgcgtt caacgcggca aacatcgcaa aagccaatag
tacagttttg atttgcatat taacggcgat tttttaaatt atcttattta
ataaatagtt atgacgccta caactccccg cccgcgttga ctcgctgcac
ctcgagcagt tcgttgacgc cttcctccgt gtggccgaac acgtcgagcg
ggtggtcgat gaccagcggc gtgccgcacg cgacgcacaa gtatctgtac
accgaatgat cgtcgggcga aggcacgtcg gcctccaagt ggcaatattg
gcaaattcga aaatatatac agttgggttg tttgcgcata tctatcgtgg
cgttgggcat gtacgtccga acgttgattt gcatgcaagc cgaaattaaa
tcattgcgat tagtgcgatt aaaacgttgt acatcctcgc ttttaatcat
gccgtcgatt aaatcgcgca atcgagtcaa gtgatcaaag tgtggaataa
tgttttcttt gtattcccga gtcaagcgca gcgcgtattt taacaaacta
gccatcttgt aagttagttt catttaatgc aactttatcc aataatatat
tatgtatcgc acgtcaagaa ttaacaatgc gcccgttgtc gcatctcaac
acgactatga tagagatcaa ataaagcgcg aattaaatag cttgcgacgc
aacgtgcacg atctgtgcac gcgttccggc acgagctttg attgtaataa
gtttttacga agcgatgaca tgacccccgt agtgacaacg atcacgccca
aaagaactgc cgactacaaa attaccgagt atgtcggtga cgttaaaact
attaagccat ccaatcgacc gttagtcgaa tcaggaccgc tggtgcgaga
agccgcgaag tatggcgaat gcatcgtata acgtgtggag tccgctcatt
agagcgtcat gtttagacaa gaaagctaca tatttaattg atcccgatga
ttttattgat aaattgaccc taactccata cacggtattc tacaatggcg
gggttttggt caaaatttcc ggactgcgat tgtacatgct gttaacggct
ccgcccacta ttaatgaaat taaaaattcc aattttaaaa aacgcagcaa
gagaaacatt tgtatgaaag aatgcgtaga aggaaagaaa aatgtcgtcg
acatgctgaa caacaagatt aatatgcctc cgtgtataaa aaaaatattg
aacgatttga aagaaaacaa tgtaccgcgc ggcggtatgt acaggaagag
gtttatacta aactgttaca ttgcaaacgt ggtttcgtgt gccaagtgtg
aaaaccgatg tttaatcaag gctctgacgc atttctacaa ccacgactcc
aagtgtgtgg gtgaagtcat gcatctttta atcaaatccc aagatgtgta
taaaccacca aactgccaaa aaatgaaaac tgtcgacaag ctctgtccgt
ttgctggcaa ctgcaagggt ctcaatccta tttgtaatta ttgaataata
aaacaattat aaatgctaaa tttgtttttt attaacgata caaaccaaac
gcaacaagaa catttgtagt attatctata attgaaaacg cgtagttata
atcgctgagg taatatttaa aatcattttc aaatgattca cagttaattt
gcgacaatat aattttattt tcacataaac tagacgcctt gtcgtcttct
tcttcgtatt ccttctcttt ttcatttttc tcctcataaa aattaacata
gttattatcg tatccatata tgtatctatc gtatagagta aatttttgt
tgtcataaat atatatgtct tttttaatgg ggtgtatagt accgctgcgc
atagtttttc tgtaatttac aacagtgcta ttttctggta gttcttcgga
gtgtgttgct ttaattatta aatttatata atcaatgaat ttgggatcgt
cggttttgta caatatgttg ccggcatagt
acgcagcttc ttctagttca attacaccat tttttagcag caccggatta
acataacttt ccaaaatgtt gtacgaaccg ttaaacaaaa acagttcacc
tccctttttct atactattgt ctgcgagcag ttgtttgttg ttaaaaataa
cagccattgt aatgagacgc acaaactaat atcacaaact ggaaatgtct

FIGURE 11 (Cont'd)

ctgtcccgat ttatttgaaa cactacaaat taaaggcgag ctttcgtacc
aacttgttag caatattatt agacagctgt gtgaagcgct caacgatttg
cacaagcaca atttcataca caacgacata aaactcgaaa atgtcttata
tttcgaagca cttgatcgcg tgtatgtttg cgattacgga ttgtgcaaac
acgaaaactc acttagcgtg cacgacggca cgttggagta ttttagtccg
gaaaaaattc gacacacaac tatgcacgtt tcgtttgact ggtacgcggc
gtgttaacat acaagttgct aacgtaatca tggtcatagc tgtttcctgt
gtgaaattgt tatccgctca caattccaca caacatacga gccggaagca
taaagtgtaa agcctggggt gcctaatgag tgagctaact cacattaatt
gcgttgcgct cactgcccgc tttccagtcg ggaaacctgt cgtgccagct
gcattaatga atcggccaac gcgcggggag aggcggtttg cgtattgggc
gctcttccgc ttcctcgctc actgactcgc tgcgctcggt cgttcggctg
cggcgagcgg tatcagctca ctcaaaggcg gtaatacggt tatccacaga
atcaggggat aacgcaggaa agaacatgtg agcaaaaggc cagcaaaagg
ccaggaaccg taaaaaggcc gcgttgctgg cgtttttcca taggctccgc
ccccctgacg agcatcacaa aaatcgacgc tcaagtcaga ggtggcgaaa
cccgacagga ctataaagat accaggcgtt tccccctgga agctccctcg
tgcgctctcc tgttccgacc ctgccgctta ccggatacct gtccgccttt
ctcccttcgg gaagcgtggc gctttctcat agctcacgct gtaggtatct
cagttcggtg taggtcgttc gctccaagct gggctgtgtg cacgaacccc
ccgttcagcc cgaccgctgc gccttatccg gtaactatcg tcttgagtcc
aacccggtaa
gacacgactt atcgccactg gcagcagcca ctggtaacag gattagcaga
gcgaggtatg taggcggtgc tacagagttc ttgaagtggt ggcctaacta
cggctacact agaaggacag tatttggtat ctgcgctctg ctgaagccag
ttaccttcgg aaaaagagtt ggtagctctt gatccggcaa acaaaccacc
gctggtagcg gtggtttttt tgtttgcaag cagcagatta cgcgcagaaa
aaaaggatct caagaagatc ctttgatctt ttctacgggg tctgacgctc
agtggaacga aaactcacgt taagggattt tggtcatgag attatcaaaa
aggatcttca cctagatcct tttaaattaa aaatgaagtt ttaaatcaat
ctaaagtata tatgagtaaa cttggtctga cagttaccaa tgcttaatca
gtgaggcacc tatctcagcg atctgtctat ttcgttcatc catagttgcc
tgactccccg tcgtgtagat aactacgata cgggagggct taccatctgg
ccccagtgct gcaatgatac cgcgagaccc acgctcaccg gctccagatt
tatcagcaat aaaccagcca gccggaaggg ccgagcgcag aagtggtcct
gcaactttat ccgcctccat ccagtctatt aattgttgcc gggaagctag
agtaagtagt tcgccagtta atagtttgcg caacgttgtt gccattgcta
caggcatcgt ggtgtcacgc tcgtcgtttg gtatggcttc attcagctcc
ggttcccaac gatcaaggcg agttacatga tcccccatgt tgtgcaaaaa
agcggttagc tccttcggtc ctccgatcgt tgtcagaagt aagttggccg
cagtgttatc actcatggtt atggcagcac tgcataattc tcttactgtc
atgccatccg taagatgctt ttctgtgact ggtgagtact caaccaagtc
attctgagaa tagtgtatgc ggcgaccgag ttgctcttgc ccggcgtcaa
tacgggataa taccgcgcca catagcagaa ctttaaaagt gctcatcatt
ggaaaacgtt cttcggggcg aaaactctca aggatcttac cgctgttgag
atccagttcg atgtaaccca ctcgtgcacc caactgatct tcagcatctt
ttactttcac cagcgtttct gggtgagcaa aaacaggaag gcaaaatgcc
gcaaaaaagg gaataagggc gacacggaaa tgttgaatac tcatactctt
cctttttcaa tattattgaa gcatttatca gggttattgt ctcatgagcg
gatacatatt tgaatgtatt tagaaaaata aacaaatagg ggttccgcgc
acatttcccc gaaaagtgcc acctgacgtc taagaaacca ttattatcat
gacattaacc tataaaaata ggcgtatcac gaggcccttt cgtctcgcgc
gtttcggtga tgacggtgaa aacctctgac acatgcagct cccggagacg
gtcacagctt gtctgtaagc ggatgccggg agcagacaag cccgtcaggg

FIGURE 11 (Cont'd)

atcaatatat agttgctgat atcatggaga taattaaaat gataaccatc
tcgcaaataa ataagtattt tactgttttc gtaacagttt tgtaataaaa
aaacctataa atattccgga ttattcatac cgtcccacca tcgggcgcgg
atcccgggta ccttctagaa ttccggagcg gccgctgcag atctgatcct
ttcctgggac ccggcaagaa ccaaaaactc actctcttca aggaaatccg
taatgttaaa cccgacacga tgaagcttgt cgttggatgg aaaggaaaag
agttctacag ggaaacttgg acccgcttca tggaagacag cttccccatt
gttaacgacc aagaagtgat ggatgttttc cttgttgtca acatgcgtcc
cactagaccc aaccgttgtt acaaattcct ggcccaacac gctctgcgtt
gcgaccccga ctatgtacct catgacgtga ttaggatcgt cgagccttca
tgggtgggca gcaacaacga gtaccgcatc agcctggcta agaagggcgg
cggctgccca ataatgaacc ttcactctga gtacaccaac tcgttcgaac
agttcatcga tcgtgtcatc tgggagaact tctacaagcc catcgtttac
atcggtaccg actctgctga agaggaggaa attctccttg aagtttccct
ggtgttcaaa gtaaaggagt ttgcaccaga cgcacctctg ttcactggtc
cggcgtatta aaacacgata cattgttatt agtacattta ttaagcgcta
gattctgtgc gttgttgatt tacagacaat tgttgtacgt attttaataa
ttcattaaat ttataatctt tagggtggta tgttagagcg aaaatcaaat
gattttcagc gtctttatat ctgaatttaa atattaaatc ctcaatagat
ttgtaaaata ggtttcgatt agtttcaaac aagggttgtt tttccgaacc
gatggctgga ctatctaatg gattttcgct caacgccaca aaacttgcca
aatcttgtag cagcaatcta gctttgtcga tattcgtttg tgttttgttt
tgtaataaag gttcgacgtc gttcaaaata ttatgcgctt ttgtatttct
ttcatcactg tcgttagtgt acaattgact cgacgtaaac acgttaaata
aagcttggac atatttaaca tcgggcgtgt tagctttatt aggccgatta
tcgtcgtcgt cccaaccctc gtcgttagaa gttgcttccg aagacgattt
tgccatagcc acacgacgcc tattaattgt gtcggctaac acgtccgcga
tcaaatttgt agttgagctt tttggaatta tttctgattg cgggcgtttt
tgggcgggtt tcaatctaac tgtgcccgat tttaattcag acaacacgtt
agaaagcgat ggtgcaggcg gtggtaacat ttcagacggc aaatctacta
atggcggcgg tggtggagct gatgataaat ctaccatcgg tggaggcgca
ggcggggctg gcggcggagg cggaggcgga ggtggtggcg gtgatgcaga
cggcggttta ggctcaaatg tctctttagg caacacagtc ggcacctcaa
ctattgtact ggtttcgggc gccgtttttg gtttgaccgg tctgagacga
gtgcgatttt tttcgtttct aatagcttcc aacaattgtt gtctgtcgtc
taaaggtgca gcgggttgag gttccgtcgg cattggtgga gcgggcggca
attcagacat cgatggtggt ggtggtggtg gaggcgctgg aatgttaggc
acgggagaag gtggtggcgg cggtgccgcc ggtataattt gttctggttt
agtttgttcg cgcacgattg tgggcaccgg cgcaggcgcc gctggctgca
caacggaagg tcgtctgctt cgaggcagcg cttggggtgg tggcaattca
atattataat tggaatacaa atcgtaaaaa tctgctataa gcattgtaat
ttcgctatcg tttaccgtgc cgatatttaa caaccgctca atgtaagcaa
ttgtattgta aagagattgt ctcaagctcg ccgcacgccg ataacaagcc
ttttcatttt tactacagca ttgtagtggc gagacacttc gctgtcgtcg
acgtacatgt atgctttgtt gtcaaaaacg tcgttggcaa gctttaaaat
atttaaaaga acatctctgt tcagcaccac tgtgttgtcg taaatgttgt
ttttgataat ttgcgcttcc gcagtatcga cacgttcaaa aaattgatgc
gcatcaattt tgttgttcct attattgaat aaataagatt gtacagattc
atatctacga ttcgtcatgg ccaccacaaa tgctacgctg caaacgctgg
tacaatttta cgaaaactgc aaaaacgtca aaactcggta taaaataatc
aacgggcgct ttggcaaaat atctatttta tcgcacaagc ccactagcaa
attgtatttg cagaaaacaa tttcggcgca caattttaac gctgacgaaa
taaaagttca ccagttaatg agcgaccacc caaattttat aaaaatctat
tttaatcacg gttccatcaa caaccaagtg atcgtgatgg actacattga

FIGURE 11 (Cont'd)

```
cgcgtcagcg ggtgttggcg ggtgtcgggg ctggcttaac tatgcggcat
cagagcagat tgtactgaga gtgcaccata tgcggtgtga aataccgcac
agatgcgtaa ggagaaaata ccgcatcagg cgccattcgc cattcaggct
gcgcaactgt tgggaagggc gatcggtgcg ggcctcttcg ctattacgcc
agctggcgaa aggggatgt gctgcaaggc gattaagttg ggtaacgcca
gggttttccc agtcacgacg ttgtaaaacg acggccagtg cc
//
```

FIGURE 12

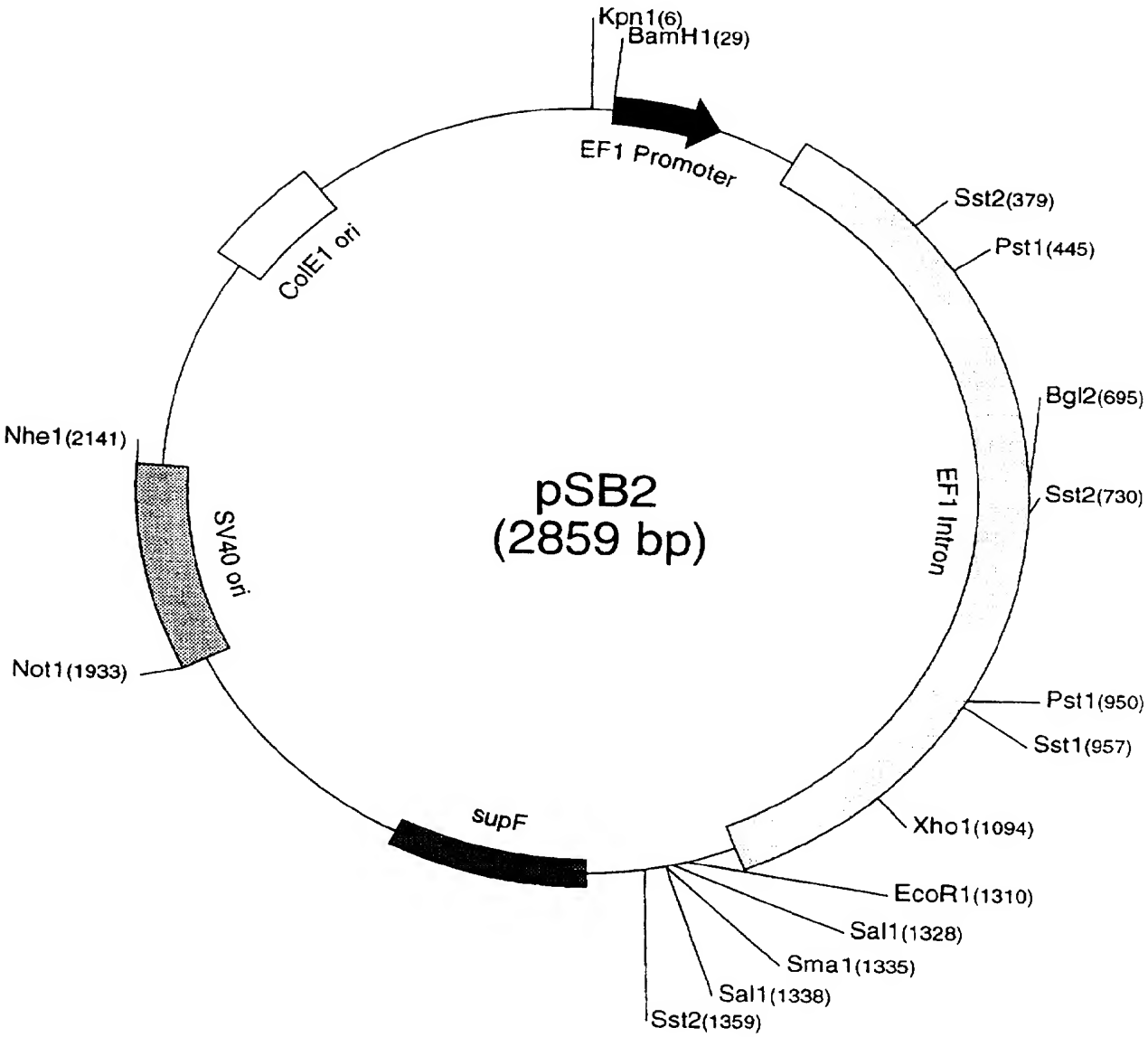

Kpn1(6)
BamH1(29)
EF1 Promoter
Sst2(379)
Pst1(445)
Bgl2(695)
Sst2(730)
EF1 Intron
Pst1(950)
Sst1(957)
Xho1(1094)
EcoR1(1310)
Sal1(1328)
Sma1(1335)
Sal1(1338)
Sst2(1359)
supF
Not1(1933)
SV40 ori
Nhe1(2141)
ColE1 ori
pSB2
(2859 bp)

FIGURE 13

Confirmation of pAP 190 purity by Western analysis

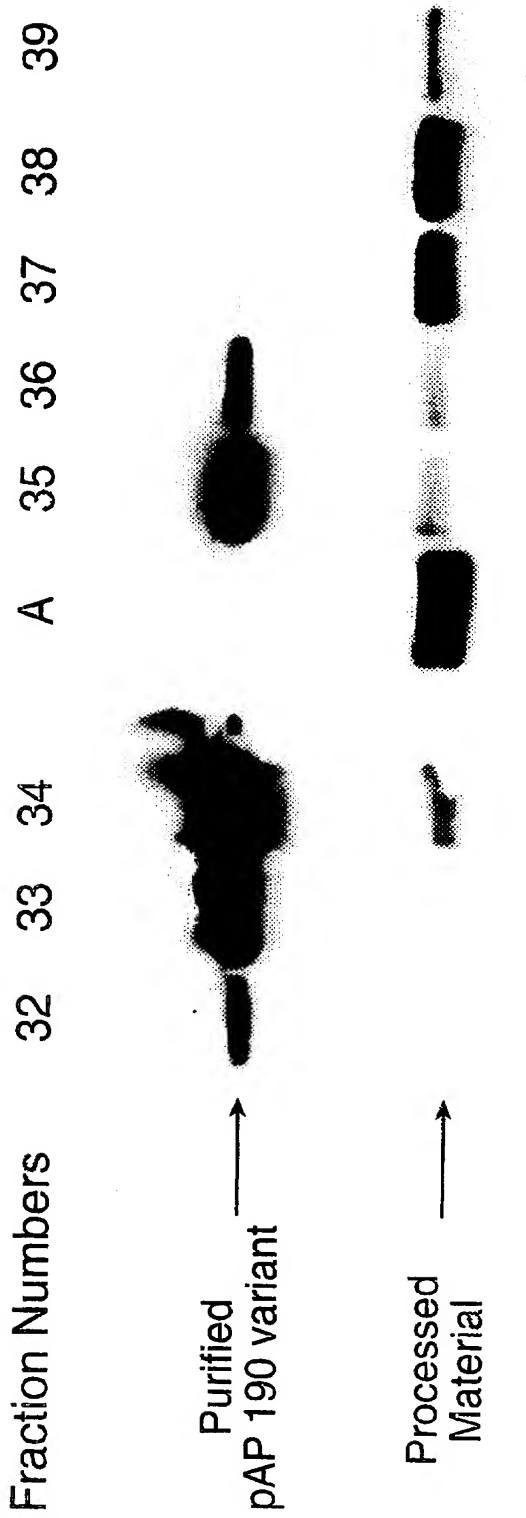
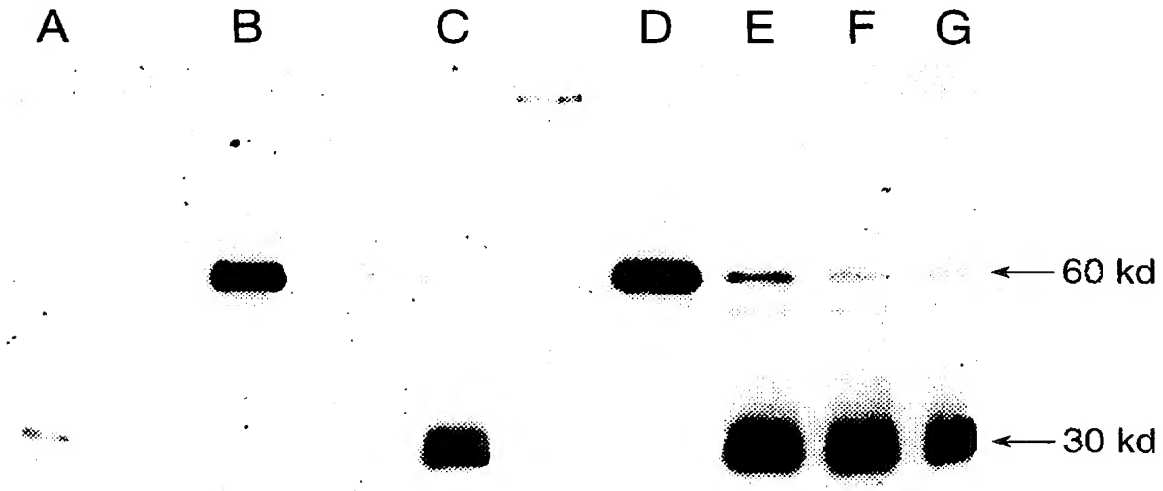


FIGURE 14

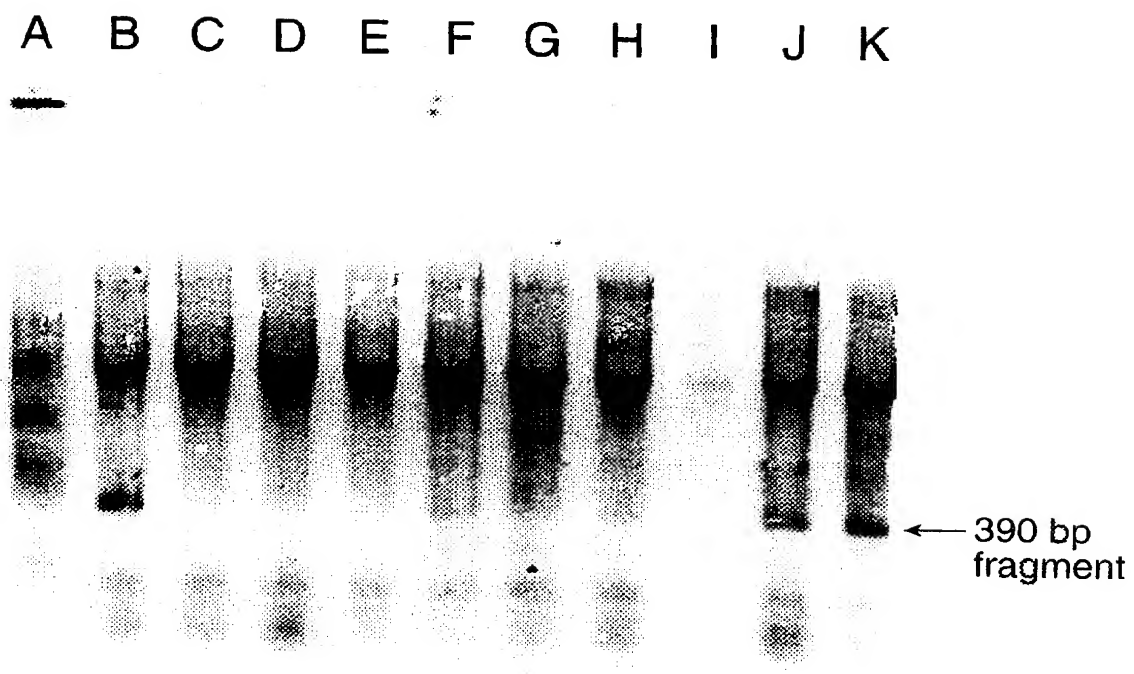
Cleavage of pAP 190 by HIV protease



A. Ricin standard
B. pAP 190
C. pAP 190 + HIV protease (3 hours)
D. pAP 190
E. pAP 190 + HIV protease (30 minutes)
F. pAP 190 + HIV protease (1 hour)
G. pAP 190 + HIV protease (2 hours)

FIGURE 15

Activation of pAP 190



A. RNA Ladder
B. Ricin A chain
C. Negative control
D. 340 pg pAP 190 variant
E. 2.1 ng pAP 190 variant
F. 12.5 ng pAP 190 variant
G. 75 ng pAP 190 variant
H. 340 pg 190 + HIV protease
I. 2.1 ng 190 + HIV protease
J. 12.5 ng 190 + HIV protease
K. 75 ng 190 + HIV protease

FIGURE 16A

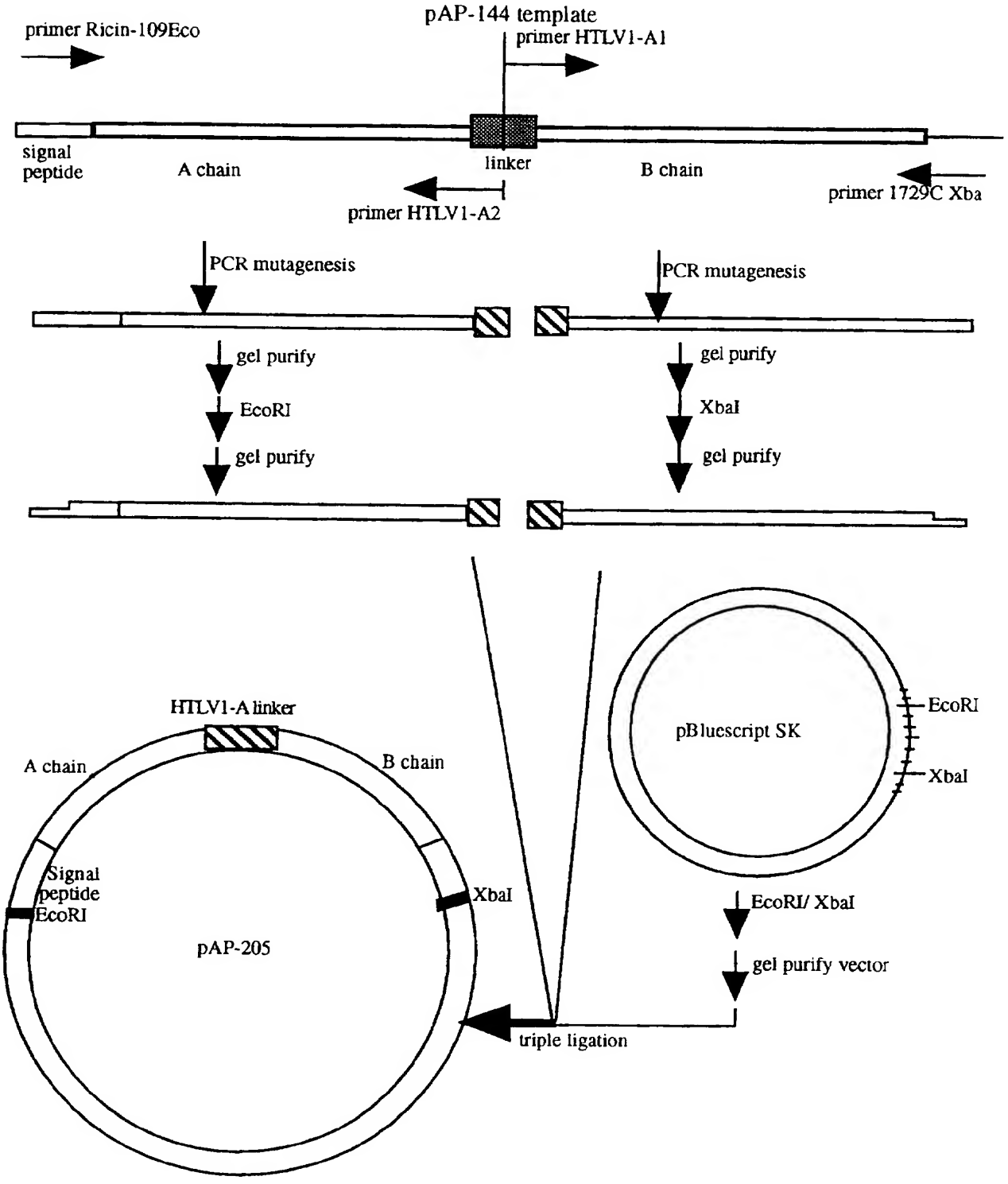

pAP-144 template
primer Ricin-109Eco
primer HTLV1-A1
signal peptide
A chain
linker
B chain
primer 1729C Xba
primer HTLV1-A2
PCR mutagenesis
PCR mutagenesis
gel purify
EcoRI
gel purify
gel purify
XbaI
gel purify
HTLV1-A linker
A chain
B chain
Signal peptide
EcoRI
XbaI
pAP-205
pBluescript SK
EcoRI
XbaI
EcoRI/ XbaI
gel purify vector
triple ligation

FIGURE 16B

WT preproricin linker
primer HTLV1-A1
5' - CCGGTGATGCATCCTAATGCTGATGTTTGT -3'
TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT
AGAAACGAATATTCCGGTCACCACGGTTTAAAATTA
3' - AGCAGTGTCAAAAGACGCGGAGTTCACGAT-5'
primer HTLV1-A2
PCR mutagenesis
ligate with pBluescript SK
pAP 205 linker
(HTLV1-A variant)
TCTGCGCCTCAAGTGCTACCGGTGATGCATCCTAAT
AGACGCGGAGTTCACGATGGCCACTACGTAGGATTA

FIGURE 16C

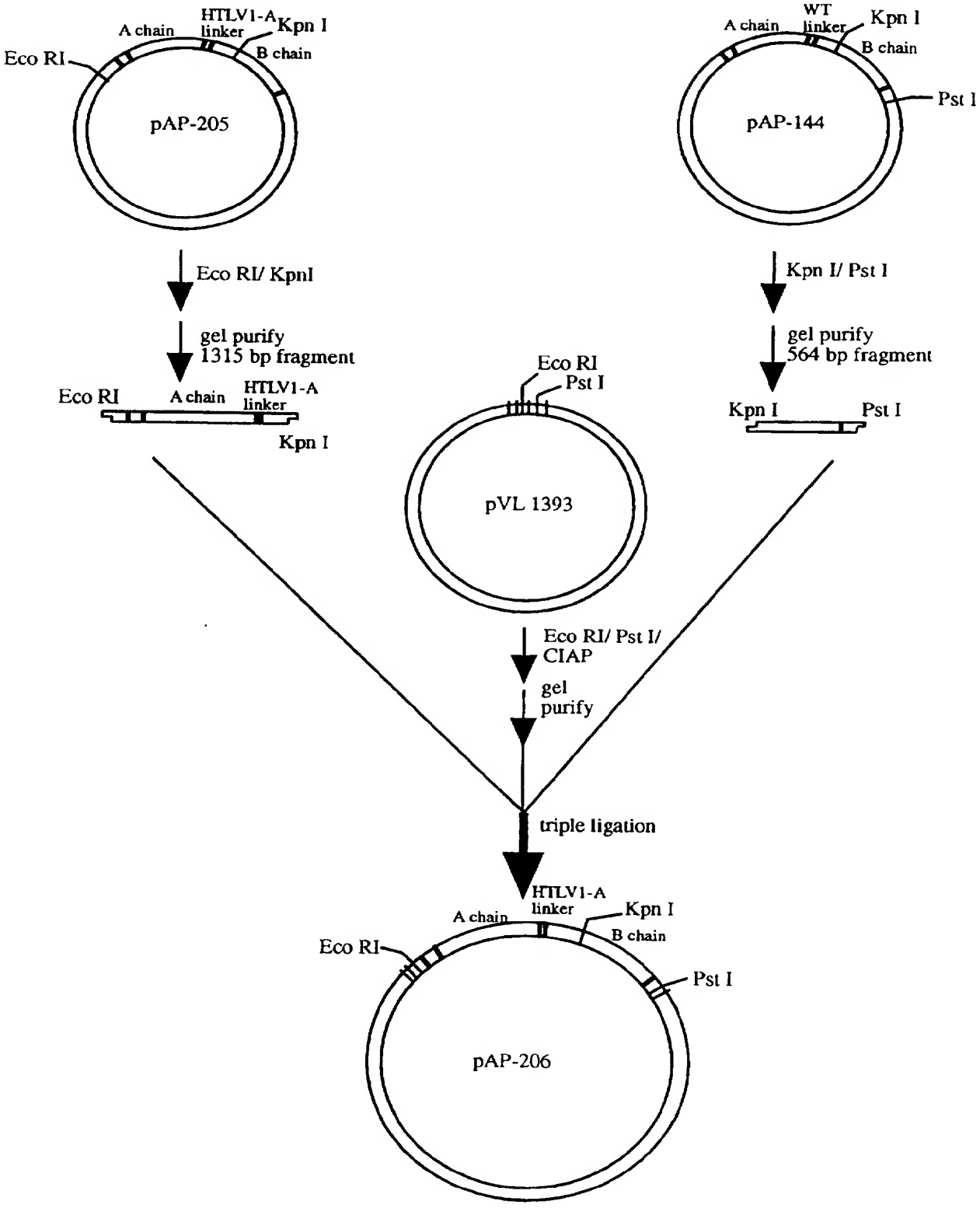

Eco RI
A chain
HTLV1-A linker
Kpn I
B chain
pAP-205
Eco RI/ KpnI
gel purify
1315 bp fragment
Eco RI
A chain
HTLV1-A linker
Kpn I
WT linker
Kpn I
A chain
B chain
Pst I
pAP-144
Kpn I/ Pst I
gel purify
564 bp fragment
Kpn I
Pst I
Eco RI
Pst I
pVL 1393
Eco RI/ Pst I/ CIAP
gel purify
triple ligation
HTLV1-A linker
A chain
Kpn I
B chain
Eco RI
Pst I
pAP-206

FIGURE 16D

```
                  10        20        30        40        50
                  |         |         |         |         |
   1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
     CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

  51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
     CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

 101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
     TCCTATTGTTGTATAAGGGGTTTGTTATGGGTTAATATTTGAAATGGTGT

 151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTCGCGG
     CGCCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

 201 TCGTTTAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTTGCCAA
     AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

 251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
     TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

 301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
     TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

 351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
     ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

 401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
     TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

 451 CGATATACATTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
     GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG

 501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
     ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

 551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAACT
     GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

 601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
     GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTC

 651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
     TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

 701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
     CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCCTCT

 751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
     GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

 801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
     AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

 851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
     ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

 901 TCGTCACAGTTTTCTGCGCCTCAAGTGCTACCGGTGATGCATCCTAATGC
     AGCAGTGTCAAAAGACGCGGAGTTCACGATGGCCACTACGTAGGATTACG

 951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
```

FIGURE 16D (CONT'D)

```
      ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC

1001  GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
      CAGATACACAACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051  CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
      GTCAACACCGGTACGTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101  GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
      CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151  GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
      CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201  ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
      TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251  CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
      GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301  TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
      AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351  AATAATACACAACCTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG
      TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401  CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
      GAACGTTCGTTTATCACCTGTTCATACCTATCTCCTGACATCGTCACTTT

1451  AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
      TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501  CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
      GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551  TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
      ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601  TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
      AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

1651  GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
      CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701  TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
      ACCACTGGGTTTGGTTTATACCAATGGTAATAAAACTATCTGTCTAATGA

1751  CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
      GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801  GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
      CCTGTAACATTTAAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851  TGCAG
      ACGTC
```

FIGURE 17A

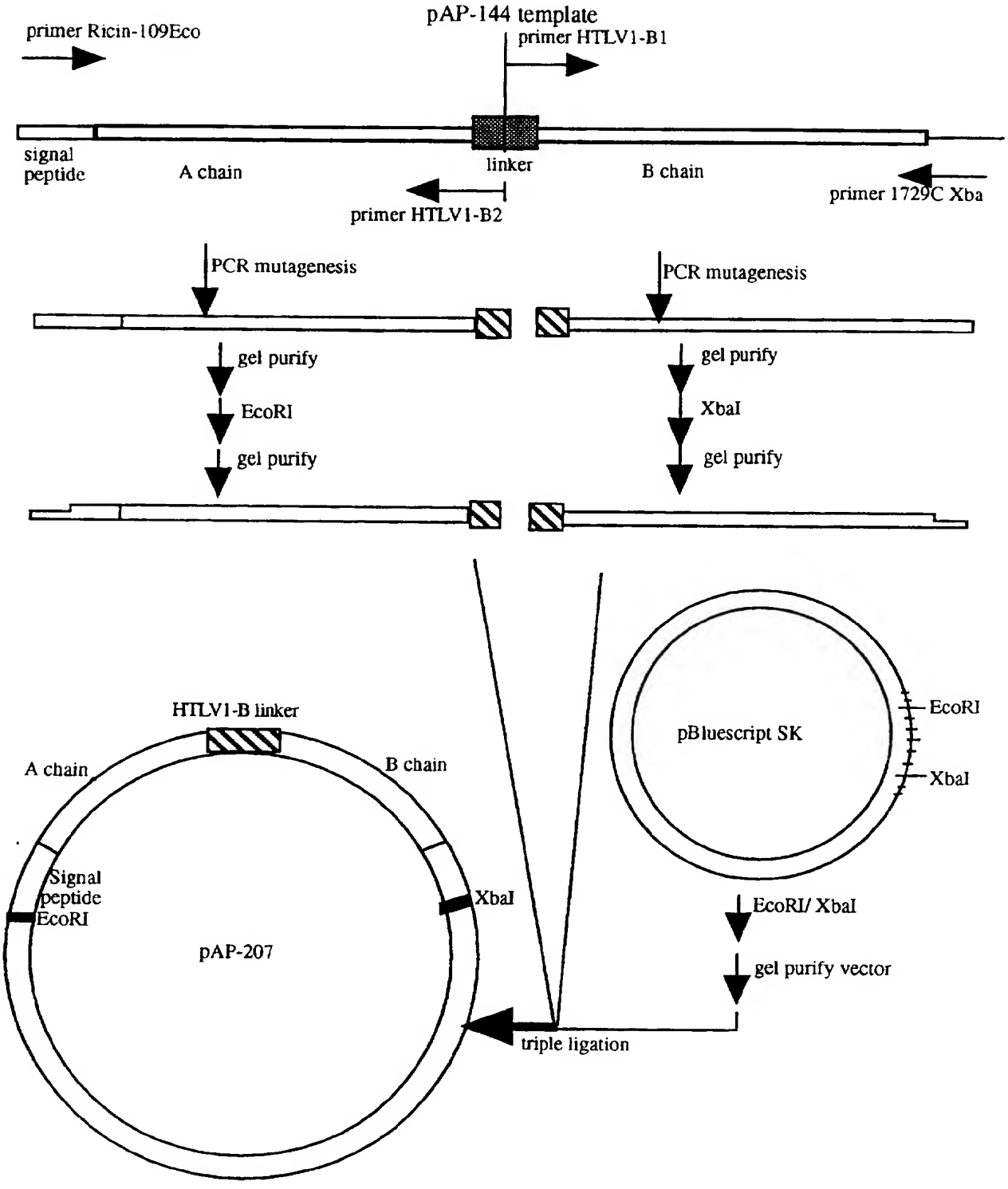

pAP-144 template
primer Ricin-109Eco
primer HTLV1-B1
signal peptide
A chain
linker
B chain
primer 1729C Xba
primer HTLV1-B2
PCR mutagenesis
PCR mutagenesis
gel purify
EcoRI
gel purify
gel purify
XbaI
gel purify
HTLV1-B linker
A chain
B chain
Signal peptide
EcoRI
XbaI
pAP-207
pBluescript SK
EcoRI
XbaI
EcoRI/ XbaI
gel purify vector
triple ligation

FIGURE 17B

WT preproricin linker

primer HTLV1-B1

5' - GTGGTGCAACCTAAGAATGCTGATGTTTGT -3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT

AGAAACGAATATTCCGGTCACCACGGTTTAAAATTA

3' - AGCAGTGTCAAAGATTCTGATTTCACGAT-5'

primer HTLV1-B2

PCR mutagenesis

ligate with pBluescript SK

pAP 207 linker
(HTLV1-B variant)

TCTAAGACTAAAGTGCTAGTGGTGCAACCTAAGAAT

AGATTCTGATTTCACGATCACCACGTTGGATTCTTA

FIGURE 17C

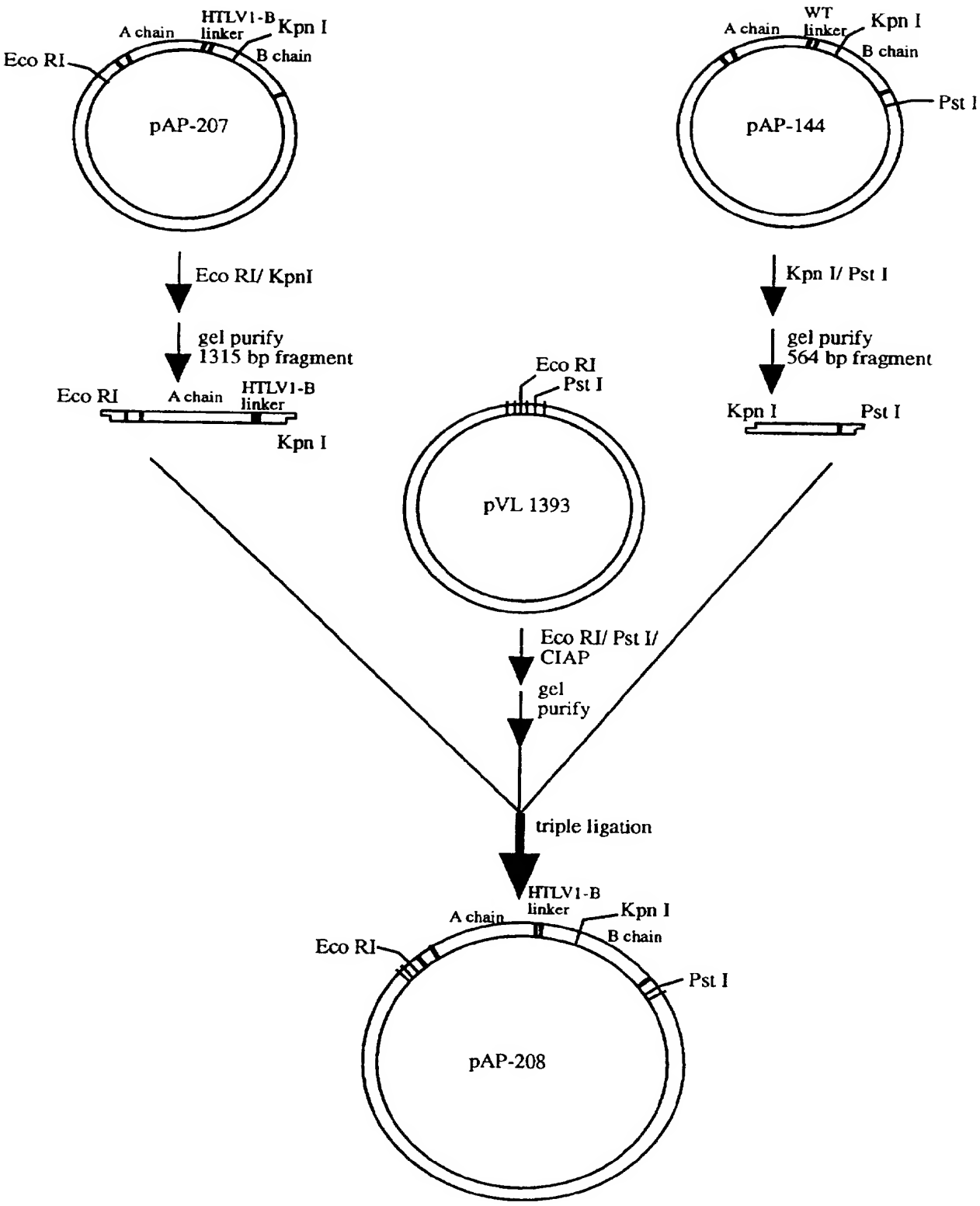

A chain
HTLV1-B linker
Kpn I
B chain
Eco RI
pAP-207
Eco RI/ KpnI
gel purify
1315 bp fragment
Eco RI
A chain
HTLV1-B linker
Kpn I
WT linker
Kpn I
A chain
B chain
Pst I
pAP-144
Kpn I/ Pst I
gel purify
564 bp fragment
Kpn I
Pst I
Eco RI
Pst I
pVL 1393
Eco RI/ Pst I/ CIAP
gel purify
triple ligation
HTLV1-B linker
A chain
Kpn I
B chain
Eco RI
Pst I
pAP-208

FIGURE 17D

```
                10        20        30        40        50
                |         |         |         |         |
  1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
    CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

 51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
    CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
    TCCTATTGTTGTATAAGGGGTTTGTTATGGGTTAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTCGCGG
    CGCCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTTGCCAA
    AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
    TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
    TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
    ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
    TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
    GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
    ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAACT
    GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
    GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTC

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
    TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
    CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
    GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
    AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
    ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTCTAAGACTAAAGTGCTAGTGGTGCAACCTAAGAATGC
    AGCAGTGTCAAAAGATTCTGATTTCACGATCACCACGTTGGATTCTTACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
    ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC
```

FIGURE 17D (CONT'D)

```
1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
     CAGATACACAACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
     GTCAACACCGGTACGTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
     CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
     CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
     TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
     GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
     AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG
     TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
     GAACGTTCGTTTATCACCTGTTCATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
     TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
     GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
     ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
     AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
     CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
     ACCACTGGGTTTGGTTTATACCAATGGTAATAAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
     GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
     CCTGTAACATTTAAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
     ACGTC
```

FIGURE 18A

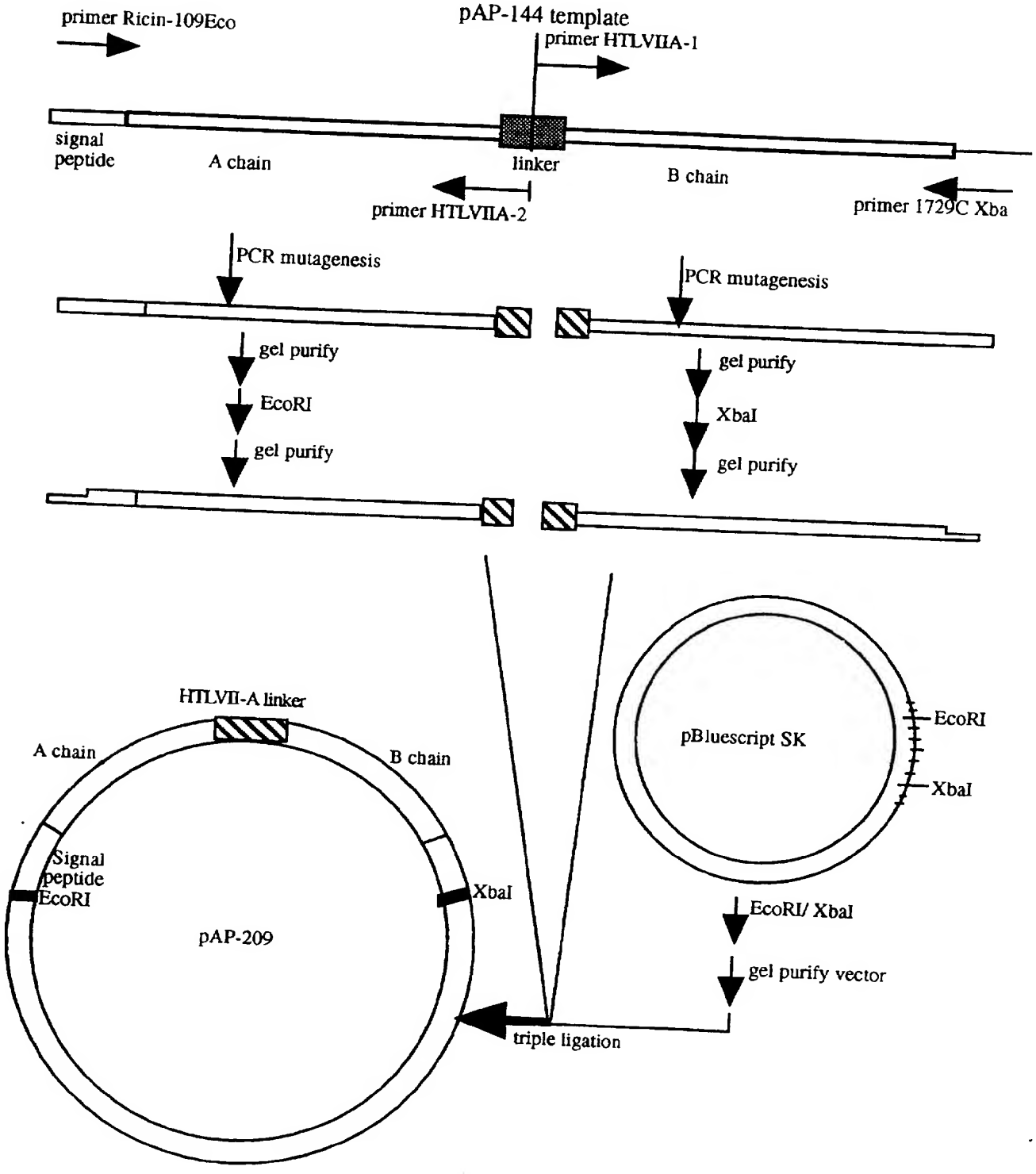

primer Ricin-109Eco
pAP-144 template
primer HTLVIIA-1
signal peptide
A chain
linker
B chain
primer HTLVIIA-2
primer 1729C Xba
PCR mutagenesis
gel purify
EcoRI
gel purify
PCR mutagenesis
gel purify
XbaI
gel purify
HTLVII-A linker
A chain
B chain
Signal peptide EcoRI
XbaI
pAP-209
pBluescript SK
EcoRI
XbaI
EcoRI/ XbaI
gel purify vector
triple ligation

FIGURE 18B

WT preproricin linker

primer HTLV11-A1

5'- GTGGTGCAACCTAGGAATGCTGATGTTTGT -3'

TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT

AGAAACGAATATTCCGGTCACCACGGTTTAAAATTA

3'- AGCAGTGTCAAAAGATTCTGATTTCACGAT-5'

primer HTLV11-A2

PCR mutagenesis

ligate with pBluescript SK

pAP 209 linker
(HTLV11-A variant)

TCTAAGACTAAAGTGCTAGTGGTGCAACCTAGGAAT

AGATTCTGATTTCACGATCACCACGTTGGATCCTTA

FIGURE 18C

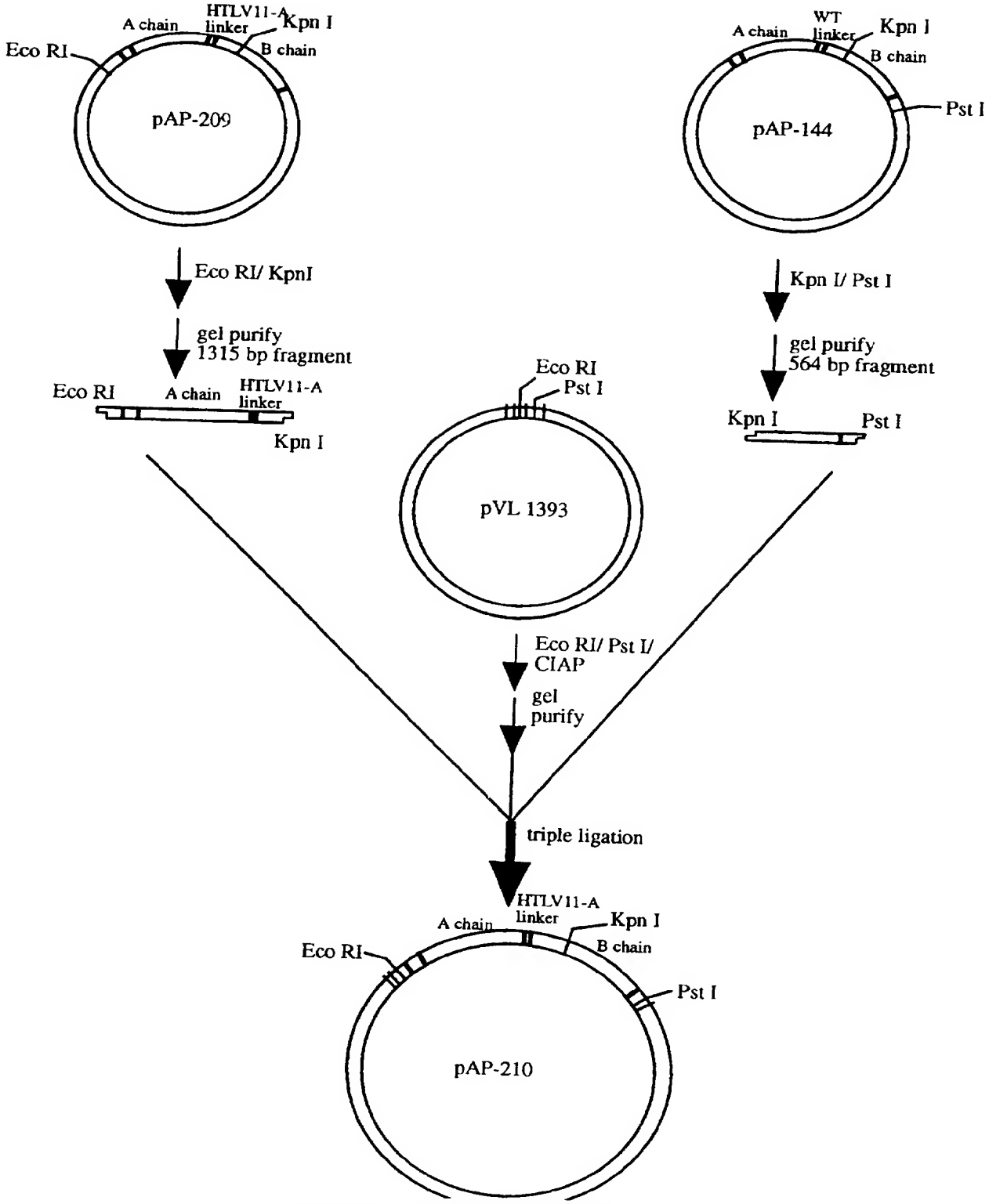

A chain
HTLV11-A linker
Kpn I
Eco RI
B chain
pAP-209
Eco RI/ KpnI
gel purify
1315 bp fragment
Eco RI
A chain
HTLV11-A linker
Kpn I
WT linker
Kpn I
A chain
B chain
Pst I
pAP-144
Kpn I/ Pst I
gel purify
564 bp fragment
Kpn I
Pst I
Eco RI
Pst I
pVL 1393
Eco RI/ Pst I/ CIAP
gel purify
triple ligation
HTLV11-A linker
A chain
Kpn I
Eco RI
B chain
Pst I
pAP-210

FIGURE 18D

```
                10        20        30        40        50
                |         |         |         |         |
  1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
    CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

 51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
    CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
    TCCTATTGTTGTATAAGGGGTTTGTTATGGGTTAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTCGCGG
    CGCCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTTGCCAA
    AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
    TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
    TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
    ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
    TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
    GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
    ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAACT
    GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
    GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTC

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
    TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
    CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
    GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
    AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
    ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTCTAAGACTAAAGTGCTAGTGGTGCAACCTAGGAATGC
    AGCAGTGTCAAAAGATTCTGATTTCACGATCACCACGTTGGATCCTTACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
    ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC
```

FIGURE 18D (CONT'D)

```
1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
     CAGATACACAACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
     GTCAACACCGGTACGTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
     CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
     CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
     TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
     GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
     AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG
     TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
     GAACGTTCGTTTATCACCTGTTCATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
     TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
     GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
     ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
     AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
     CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
     ACCACTGGGTTTGGTTTATACCAATGGTAATAAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
     GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
     CCTGTAACATTTAAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
     ACGTC
```

FIGURE 19A

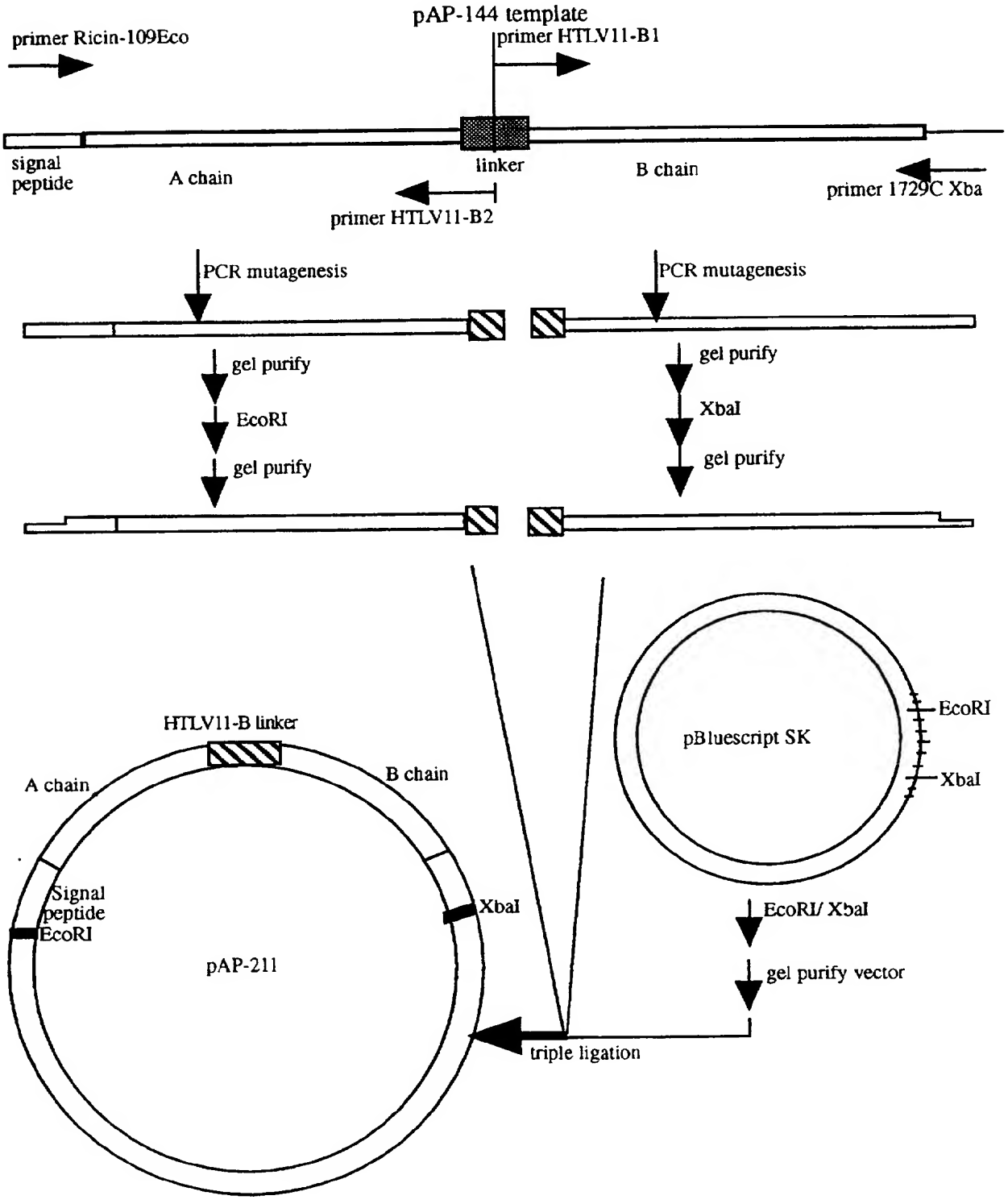

pAP-144 template
primer Ricin-109Eco
primer HTLV11-B1
signal peptide
A chain
linker
B chain
primer 1729C Xba
primer HTLV11-B2
PCR mutagenesis
PCR mutagenesis
gel purify
EcoRI
gel purify
gel purify
XbaI
gel purify
HTLV11-B linker
A chain
B chain
Signal peptide
EcoRI
XbaI
pAP-211
pBluescript SK
EcoRI
XbaI
EcoRI/ XbaI
gel purify vector
triple ligation

FIGURE 19B

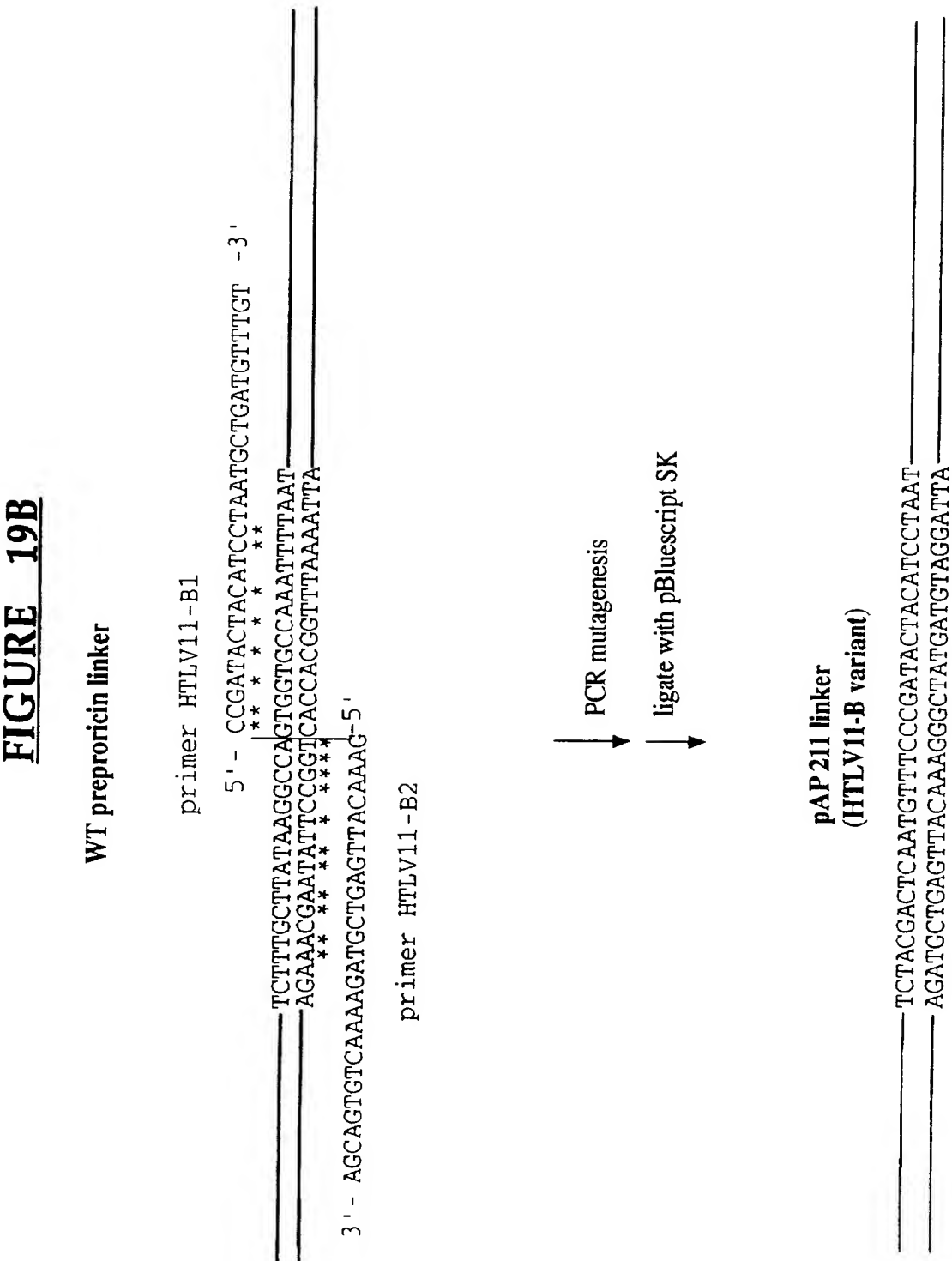

WT preproricin linker
primer HTLV11-B1
5'- CCGATACTACATCCTAATGCTGATGTTTGT -3'
TCTTTGCTTATAAGGCCAGTGGTGCCAAATTTTAAT
AGAAACGAATATTCCGGTCACCACGGTTTAAAATTA
3'- AGCAGTGTCAAAGATGCTGAGTTACAAAG-5'
primer HTLV11-B2
PCR mutagenesis
ligate with pBluescript SK
pAP211 linker
(HTLV11-B variant)
TCTACGACTCAATGTTTCCCGATACTACATCCTAAT
AGATGCTGAGTTACAAAGGGCTATGATGTAGGATTA

FIGURE 19C

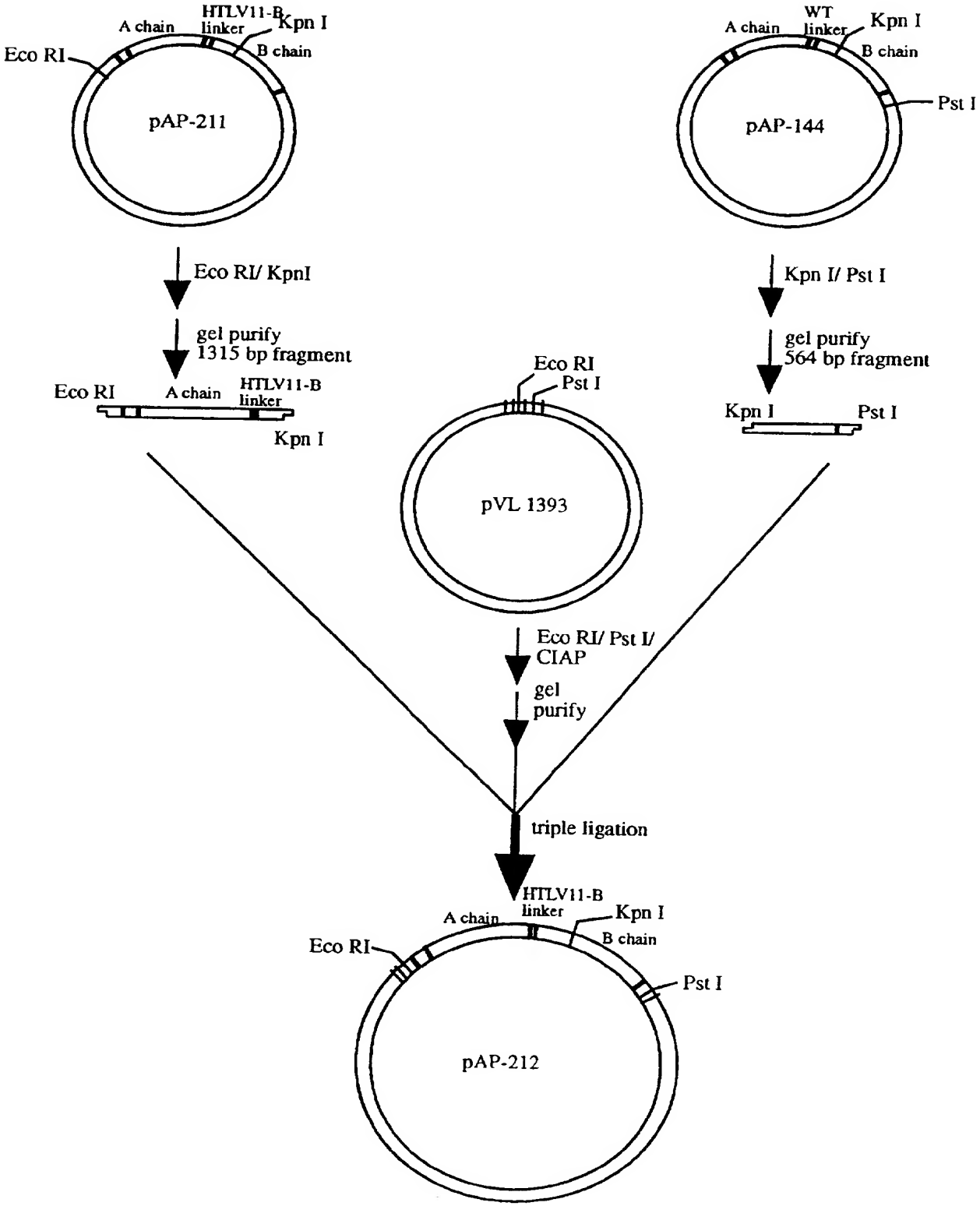

HTLV11-B
A chain
linker
Kpn I
B chain
Eco RI
pAP-211
Eco RI/ KpnI
gel purify
1315 bp fragment
Eco RI
A chain
HTLV11-B
linker
Kpn I
WT
A chain
linker
Kpn I
B chain
Pst I
pAP-144
Kpn I/ Pst I
gel purify
564 bp fragment
Kpn I
Pst I
Eco RI
Pst I
pVL 1393
Eco RI/ Pst I/
CIAP
gel
purify
triple ligation
HTLV11-B
A chain
linker
Kpn I
B chain
Eco RI
Pst I
pAP-212

FIGURE 19D

```
                10        20        30        40        50
                |         |         |         |         |
  1 GAATTCATGAAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGT
    CTTAAGTACTTTGGCCCTCCTTTATGATAACATTATACCTACATACGTCA

 51 GGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAG
    CCGTTGTACCGAAACAAAACCTAGGTGGAGTCCCACCAGAAAGTGTAATC

101 AGGATAACAACATATTCCCCAAACAATACCCAATTATAAACTTTACCACA
    TCCTATTGTTGTATAAGGGGTTTGTTATGGGTTAATATTTGAAATGGTGT

151 GCGGGTGCCACTGTGCAAAGCTACACAAACTTTATCAGAGCTGTTCGCGG
    CGCCCACGGTGACACGTTTCGATGTGTTTGAAATAGTCTCGACAAGCGCC

201 TCGTTTAACAACTGGAGCTGATGTGAGACATGATATACCAGTGTTGCCAA
    AGCAAATTGTTGACCTCGACTACACTCTGTACTATATGGTCACAACGGTT

251 ACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCA
    TGTCTCAACCAAACGGATATTTGGTTGCCAAATAAAATCAACTTGAGAGT

301 AATCATGCAGAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATA
    TTAGTACGTCTCGAAAGACAATGTAATCGCGACCTACAGTGGTTACGTAT

351 TGTGGTCGGCTACCGTGCTGGAAATAGCGCATATTTCTTTCATCCTGACA
    ACACCAGCCGATGGCACGACCTTTATCGCGTATAAAGAAAGTAGGACTGT

401 ATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGATGTTCAAAAT
    TAGTCCTTCTACGTCTTCGTTAGTGAGTAGAAAAGTGACTACAAGTTTTA

451 CGATATACATTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGC
    GCTATATGTAAGCGGAAACCACCATTAATACTATCTGAACTTGTTGAACG

501 TGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGG
    ACCATTAGACTCTCTTTTATAGCTCAACCCTTTACCAGGTGATCTCCTCC

551 CTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAACT
    GATAGAGTCGCGAAATAATAATGTCATGACCACCGTGAGTCGAAGGTTGA

601 CTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAG
    GACCGAGCAAGGAAATATTAAACGTAGGTTTACTAAAGTCTTCGTCGTTC

651 ATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGA
    TAAGGTTATATAACTCCCTCTTTACGCGTGCTCTTAATCCATGTTGGCCT

701 GATCTGCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGA
    CTAGACGTGGTCTAGGATCGCATTAATGTGAACTCTTATCAACCCCCTCT

751 CTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTTGCTAGTCCAAT
    GAAAGGTGACGTTAAGTTCTCAGATTGGTTCCTCGGAAACGATCAGGTTA

801 TCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGTA
    AGTTGACGTTTCTGCATTACCAAGGTTTAAGTCACACATGCTACACTCAT

851 TATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCA
    ATAATTAGGGATAGTATCGAGAGTACCACATATCTACGCGTGGAGGTGGT

901 TCGTCACAGTTTTCTACGACTCAATGTTTCCCGATACTACATCCTAATGC
    AGCAGTGTCAAAAGATGCTGAGTTACAAAGGGCTATGATGTAGGATTACG

951 TGATGTTTGTATGGATCCTGAGCCCATAGTGCGTATCGTAGGTCGAAATG
    ACTACAAACATACCTAGGACTCGGGTATCACGCATAGCATCCAGCTTTAC
```

FIGURE 19D (CONT'D)

```
1001 GTCTATGTGTTGATGTTAGGGATGGAAGATTCCACAACGGAAACGCAATA
     CAGATACACAACTACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTAT

1051 CAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTCTGGACTTT
     GTCAACACCGGTACGTTCAGATTATGTCTACGTTTAGTCGAGACCTGAAA

1101 GAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACG
     CTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGC

1151 GGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCA
     CCATGTCAGGCCCTCAGATACACTACTAGATACTAACGTTATGACGACGT

1201 ACTGATGCCACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCC
     TGACTACGGTGGGCGACCGTTTATACCCTATTACCTTGGTAGTATTTAGG

1251 CAGATCTAGTCTAGTTTTAGCAGCGACATCAGGGAACAGTGGTACCACAC
     GTCTAGATCAGATCAAAATCGTCGCTGTAGTCCCTTGTCACCATGGTGTG

1301 TTACAGTGCAAACCAACATTTATGCCGTTAGTCAAGGTTGGCTTCCTACT
     AATGTCACGTTTGGTTGTAAATACGGCAATCAGTTCCAACCGAAGGATGA

1351 AATAATACACAACCTTTTGTTACAACCATTGTTGGGCTATATGGTCTGTG
     TTATTATGTGTTGGAAAACAATGTTGGTAACAACCCGATATACCAGACAC

1401 CTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAAA
     GAACGTTCGTTTATCACCTGTTCATACCTATCTCCTGACATCGTCACTTT

1451 AGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAG
     TCCGACTTGTTGTCACCCGAGAAATACGTCTACCAAGTTATGCAGGAGTC

1501 CAAAACCGAGATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGT
     GTTTTGGCTCTATTAACGGAATGTTCACTAAGATTATATGCCCTTTGTCA

1551 TGTTAAGATCCTCTCTTGTGGCCCTGCATCCTCTGGCCAACGATGGATGT
     ACAATTCTAGGAGAGAACACCGGGACGTAGGAGACCGGTTGCTACCTACA

1601 TCAAGAATGATGGAACCATTTTAAATTTGTATAGTGGATTGGTGTTAGAT
     AGTTCTTACTACCTTGGTAAAATTTAAACATATCACCTAACCACAATCTA

1651 GTGAGGCGATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCA
     CACTCCGCTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGT

1701 TGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTACT
     ACCACTGGGTTTGGTTTATACCAATGGTAATAAAACTATCTGTCTAATGA

1751 CTCTTGCAGTGTGTGTGTCCTGCCATGAAAATAGATGGCTTAAATAAAAA
     GAGAACGTCACACACACAGGACGGTACTTTTATCTACCGAATTTATTTTT

1801 GGACATTGTAAATTTTGTAACTGAAAGGACAGCAAGTTATATCGAATTCC
     CCTGTAACATTTAAAACATTGACTTTCCTGTCGTTCAATATAGCTTAAGG

1851 TGCAG
     ACGTC
```

FIGURE 20

Wild type Ricin linker:	A chain- S L L I R P V V P N F N -B chain
pAP-205 linker: pAP-206 (HTLV1-A linker)	A chain- S A P Q V L P V M H P N -B chain
pAP-207 linker: pAP-208 (HTLV1-B linker)	A chain- S K T K V L V V Q P K N -B chain
pAP-209 linker: pAP-210 (HTLV11-A linker)	A chain- S I R K I L F L D G I N -B chain
pAP-211 linker: pAP-212 (HTLV11-B linker)	A chain- S T T Q C F P I L H P N -B chain

INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/CA 97/00288

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/29 C12N15/62 C12N15/70 C12N15/86 A61K38/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WESTBY M ET AL: "PREPARATION AND CHARACTERIZATION OF RECOMBINANT PRORICIN CONTAINING AN ALTERNATIVE PROTEASE-SENSITIVE LINKER SEQUENCE" BIOCONJUGATE CHEMISTRY, vol. 3, no. 5, 1 January 1992, pages 375-381, XP000578216 cited in the application see the whole document ---	1-28
Y	LEPPLA S. ET AL.: "Development of anthrax-toxin based fusion proteins for targeting of HIV-1-infected cells" ZENTRALBLATT FÜR BAKTERIOLOGIE, vol. 24, 1994, pages 431-442, XP002041056 see the whole document --- -/--	1-28

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
1 October 1997	15. 10. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kania, T

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/CA 97/00288

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PETTIT S. ET AL.: "Analysis of retroviral protease cleavage sites reveals two types of cleavage sites and the structural requirements of the P1 amino acid" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 266, no. 22, 5 August 1991, pages 14539-14547, XP002041058 cited in the application see the whole document ---	5,8,18, 20
A	EP 0 466 222 A (DOWELANCO) 15 January 1992 cited in the application see the whole document ---	1-28
A	O'HARE M. ET AL.: "Cytotoxicity of a recombinant ricin-A-chain fusion protein containing a proteolytically-cleavable spacer sequence" FEBS LETTERS, vol. 273, no. 1,2, 29 October 1990, pages 200-204, XP002041057 cited in the application see the whole document ---	1-28
A	COOK J P ET AL: "BIOLOGICALLY ACTIVE INTERLEUKIN 2-RICIN A CHAIN FUSION PROTEINS MAY REQUIRE INTRACELLULAR PROTEOLYTIC CLEAVAGE TO EXHIBIT A CYTOTOXIC EFFECT" BIOCONJUGATE CHEMISTRY, vol. 4, no. 6, 1 November 1993, pages 440-447, XP000417282 see the whole document ---	1-28
A	WO 89 01037 A (CETUS CORP) 9 February 1989 see the whole document -----	1-28

1

INTERNATIONAL SEARCH REPORT

International application No
PCT/CA 97/00288

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 21-24
is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees

INTERNATIONAL SEARCH REPORT

information on patent family members

Inte· onal Application No
PCT/CA 97/00288

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0466222 A	15-01-92	US 5248606 A	28-09-93
		AU 638133 B	17-06-93
		AU 7832991 A	12-12-91
		CA 2044201 A	12-12-91
		CN 1062172 A	24-06-92
		JP 4279599 A	05-10-92
		US 5635384 A	03-06-97
		US 5646026 A	08-07-97
WO 8901037 A	09-02-89	AU 2136788 A	01-03-89